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Address of the President
Sir Robert Robinson, at the
Anniversary Meeting, 1 December 1947

The names that have just been recounted include those of many outstanding personalities in the scientific world and it would not be fitting to attempt even brief appreciations of their manifold services on this occasion. An exception must, however, be made when we mourn such giants as two of the deceased Fellows

Sir Frederick Gowland Hopkins, O.M., was elected a Fellow of the Society in 1905; he delivered the Croonian Lecture in 1915, was Royal Medallist in 1918 and Copley Medallist in 1926. He was President of the Society from 1930 to 1935. Such are the bare facts, and though we are proud of his intimate association with the Royal Society, we do not now think of a Lecturer, a Medallist, or even of a President. Our memory dwells rather on the lovable qualities and magnanimous spirit of a devoted teacher and leader, and on the influence of his generous help to others as well as of his personal achievements during almost seventy years of scientific life. He was early imbued with the conviction that the chemistry of the living cell was *his* subject, that it was not only of transcendent importance, but also that it was ripe for development He dedicated himself to the quest and embarked with enthusiasm on a pioneering voyage of discovery. The outcome of his courage and industry was the foundation of a new scientific discipline, if not of a new science He was the father of modern schools of biochemistry and was the greatest biochemist of his generation.

Professor Max Karl Ernst Ludwig Planck was elected a Foreign Member in 1926 and was Copley Medallist for 1929. Originally a disciple of Boltzmann in the field of thermodynamics, his most characteristic contribution arose from a thorough examination of the experimental data relating to black-body radiation. His intuitive insight, powers of analysis, and willingness to accept the logic of his deductions however strange the result, made possible the transition to a new system of mechanics. It is probable that the historian of the future will date the modern era of theoretical physics from Planck's recognition of the quantum of energy, one of the most fundamental discoveries ever made in physical science.

In temperament and interests, Hopkins and Planck had little in common, but they were both the founders of schools of research, they were both great teachers and great men whose memory will long be cherished by their pupils and colla-borators.

Awards of Medals, 1947

The COPLEY MEDAL is awarded to Emeritus Professor GODFREY HAROLD HARDY in recognition of his distinguished contributions to mathematics.

For many years G. H. Hardy has occupied an outstanding position among mathematicians in this country, whilst his eminent services and original work

have also been very widely recognized abroad. In his memoirs and papers published with collaborators, especially J. E. Littlewood, are to be found the origins of the major developments of 'classical analysis' throughout the world in the years 1910 to 1935.

Among the most influential and original of his works are those dealing with the Tauberian theorem for Cesaro summability (1910); the proof that the Riemann zeta-function has an infinity of zeros on the critical line (1914); the asymptotic formula for the number of partitions of an integer (with Ramanujan, 1918); the papers on Waring's problem and Goldbach's problem (with Littlewood, 1920, 1922); the paper in *Acta Mathematica* on Fourier series (with Littlewood, 1930). He has made important contributions to lattice-point theory, to the theory of integral equations, and (with Littlewood) to the theory of Diophantine approximation.

Lying in widely separated domains of analysis, all these papers contain fundamentally novel ideas which were the starting points for new significant developments by Hardy and others. For example, a part of the distinguished work of Vinogradoff may justly be said to derive from that of Hardy. His influence has been exerted, not only by his publication of original work, but also through a long succession of pupils, many of whom are now well-known mathematicians. At a time when the vigorous treatment of analysis in University courses had still to win general acceptance, Hardy's enthusiastic advocacy and his inspiring lectures had a decisive effect   In addition, the appearance of his highly individual text-book *Pure Mathematics* helped to demolish finally the barriers between the English and Continental schools of mathematics.

It is impossible to characterize in a few sentences the achievement, so varied, and so abstruse, of the Copley Medallist for 1947 but in very ordinary words it may be said that Hardy saw clearly what were the most important tasks and problems lying before mathematicians; he had good judgment in selecting those which seemed ripe for attack, and he displayed the greatest skill and energy in their solution.

A Royal Medal is awarded to Professor Cyril Norman Hinshelwood for his outstanding researches in chemical kinetics.

Professor Hinshelwood has brought a characteristic lucidity of mind and directness of approach to bear on the investigation of many classical problems presented by the course of chemical change. He has clarified these subjects to an extent which seems remarkable to those who recall the mystery which enshrouded them only a few years ago.

The acute suggestion of Lord Cherwell relating to the consequences flowing from the relative rates of formation and decomposition of an intermediate additive complex was taken up and extended by Hinshelwood. He predicted that on the basis of this theory certain apparently monomolecular reactions should follow a bimolecular law at low pressures. One example of the justification of this pro-

phecy was found in the decomposition of acetaldehyde. This experimental verification of the hypothesis of intermediate complexes of definite life, and the new light shed on the mechanism of activation of molecules, are pillars of modern chemical kinetics. Hinshelwood has enlarged our knowledge of this subject by many other researches of great importance including those on the synthesis of water, a classical problem of seeming simplicity and real complexity and those on the conditions of 'chain' reactions. His ingenious experimental methods, though characterized by a lack of unnecessary elaborations, have nevertheless always been able to provide accurate results.

It was a very original conception to apply the principles and methods of chemical kinetics to the growth of bacteria and Hinshelwood has already made noteworthy progress in this field.

The outcome has been to provide a new and more delicate assessment of the presence and activity of factors that influence growth at all stages under defined conditions.

During the war he carried out important researches on the absorption of gases by charcoal, and on the preparation of suitable adsorbents for different purposes, ranging from gas-masks to the production of penicillin.

The brilliant exposition of chemical topics contained in Professor Hinshelwood's books places contemporary chemists and students still further in his debt. His memorable addresses on the wider implications of chemistry made as President of the Chemical Society and on the occasion of the celebration of its Centenary were, generally acclaimed. Possessing enviable linguistic attainments he has frequently been an Ambassador of British Science. Recognizing these achievements we wish to honour Professor Hinshelwood above all for his pioneering work in the laboratory and in the study, which has thrown so much light on the most fundamental processes of chemistry, the mode of interaction of molecules.

A ROYAL MEDAL is awarded to Dr FRANK MACFARLANE BURNET for his distinguished contributions to our knowledge of the viruses.

Dr Burnet, Director of the Walter and Eliza Hall Institute, at Melbourne, has made a series of extremely significant investigations of bacteriophages and viruses since 1925. In twenty-seven papers on the first topic he has made greater advances than anyone apart from d'Herelle. He has elucidated the phenomenon of lysogenesis or 'carrying' of a bacteriophage, active against other bacteria, by an organism which is itself apparently unaffected. He has shown how study, partly on a biochemical basis, of the range of activity of 'phages against various bacteria can be used to classify not only the 'phages but the bacteria themselves. Other investigations have made an important contribution to our present ideas as to what bacteriophages are.

Since 1933 he has studied many viruses pathogenic for vertebrates. He has shown how psittacosis is endemic in wild Australian parrots, and has thrown light on the epidemiology of herpes and poliomyelitis. To modern workers he is best

known for his development of several techniques for cultivating viruses in living chick embryos and their application in immunological and other studies of viruses, especially influenza. He has written monographs on the use of chick embryos in virus research, on immunity to viruses, on influenza and on the nature of antibodies.

At present he is examining the agglutination of mammalian and avian red blood cells by a number of viruses and virus-products and is thus throwing fresh light on the reactions taking place between viruses and the surfaces of susceptible cells. He has found that an enzyme produced by cholera vibrios has an action on blood cells which is quite similar to that of influenza viruses, and is capable of blocking the union between virus and susceptible cell. This discovery indicates one line of approach to the problem of the chemotherapy or chemoprophylaxis of virus infections.

Burnet has been active in many other fields, he has studied staphylococcal toxins and he was the first to make a formolized staphylococcal toxoid. He has demonstrated that a rickettsia is the cause of Australian Q-fever.

Whilst his experimental studies have demanded close attention to detail, certain continuous threads have connected his interests. The breadth of his outlook and his philosophic attitude of mind are well exemplified in his fascinating book *Biological Aspects of Infectious Diseases* and in the more recent publication *Viruses as Organisms*.

The DAVY MEDAL is awarded to Professor LINUS PAULING in recognition of his distinguished researches on molecular structure.

The unusual breadth of Professor Pauling's scientific knowledge, his mastery of physics and mathematics in addition to chemistry, made him peculiarly fitted to apply quantum mechanics to problems of chemical valency bonds In him, theoretical acumen and penetrating insight, are associated with experimental skill of the highest order and thus his theories were often tested after the devising of new physical techniques.

This combination of theoretical and practical attainments is very remarkable at such a level in both aspects.

As a natural consequence of his catholic interests Pauling's work has covered a very wide range of chemical structural problems. He has devoted much attention to line spectra and many other physical topics but his most individual achievements are in the advance of molecular structure theory. In elucidating the fundamental structure of simple organic compounds he developed the method of localized pairs (atomic orbitals) to provide a theoretical treatment of the valency conditions of such molecules. By this introduction of orbital hybridization he gave the first theoretical account of the tetrahedral distribution of the valencies of the saturated carbon atom (1928). This was followed by the introduction of the quantum mechanical concept that the actual state of a molecule such as benzene should be represented as a resonance hybrid of several valency bond structures

(1931). The application of this idea and the experimental verification of its structural implications in terms of increased stability and decreased bond distances, greatly increased our understanding of aromatic and general chemistry.

The calculus of the resonance theory of Pauling was the first to introduce a quantitative treatment of valency problems and its effect has been felt far beyond the bounds of the experimental material

In 1939 his extensive work was summarized in a great book *The Nature of the Chemical Bond* which will always be one of the classics of chemistry. Here we find a general classification of stubborn aspects of valency theory along with valuable tables of generalized and correlated data on resonance energy terms, bond distances and angles, and atomic radii.

Proceeding to apply his methods to the study of more complex molecular systems, Pauling has made important contributions to our understanding of the metallic bond; of the structure and mode of denaturation of native proteins, and of the mechanism of the fundamental reactions of immunology, that is, antibody formation, and the antibody-antigen interaction.

Professor Pauling has earned our gratitude, not only for his personal services, great as those have been, but also because he has been a focus of activity and a constant source of inspiration to other workers.

The BUCHANAN MEDAL is awarded to Sir EDWARD MELLANBY, K C.B., for his services to medical science and in recognition of his outstanding researches on dietary factors.

The Buchanan Medal is awarded every five years in respect of distinguished services to hygienic science or practice in the direction either of original research, or of professional, administrative, or constructive work, without limit of sex or nationality.

In each of these respects the award to Sir Edward Mellanby is surely one of the most appropriate that could be made. For some thirteen years he has devoted himself to the service of medical science and the national organization of medical research.

His personal research work, which has been continued right up to the present time, has been concerned mainly with problems of nutrition, and has had important results in the relation of diet to the public health.

His two most important contributions have been on the effects of deficiency of vitamin D and of vitamin A in the diet He first showed that rickets was unquestionably due to the lack of a fat-soluble vitamin, since known as vitamin D, and he also demonstrated that other factors, dietary and general, such as lack of exercise, contributed to aggravate the condition. Quite recently he has shown that lack of vitamin A, in addition to the already recognized effects on general health, is capable of producing profound neurological disorders, owing to the mechanical effect of abnormal growth of bones around the foramina through which nerves leave the central nervous system. The knowledge which has been made available

as a result of his investigations has played an important part in the feeding of the nation on scientific lines, in war, and in peace-time.

The study which he made of the effects of lack of iodine have thrown much light on the various forms of goitre, and especially on the hyperplastic and colloid forms: the former he showed to be caused by iodine deficiency, particularly in young animals, or in ante-natal states, while the latter is produced by the belated admission of iodine to such sufferers from the hyperplastic condition. The knowledge has been of value in connexion with the lack of iodine in the water and soil of some districts.

Mellanby's researches on the physiological effects of alcohol have also provided valuable knowledge concerning the effects of this substance as a constituent of the diet.

His most recent contribution has been to show that bread which is made from flour bleached by the Agene process, that is, with nitrogen trichloride, can cause the condition in dogs known as canine hysteria.

As the Secretary of the Medical Research Council, Sir Edward has proved himself a wise, strong and withal a helpful administrator.

The HUGHES MEDAL is awarded to Professor JEAN FRÉDÉRIC JOLIOT in recognition of his outstanding researches in nuclear chemistry and physics.

Professor Joliot, Director of the Nuclear Chemistry Laboratory of the Collège de France, was early concerned with investigations leading to the discovery of the neutron and with subsequent work on that particle. In 1932 he found that the very penetrating radiation emitted when beryllium is bombarded with alpha-particles was able to eject protons from hydrogenous material upon which it fell. This observation was followed up by Chadwick, who was able to show that the radiation consisted of the neutrons which he and Rutherford had sought for years.

In 1934 Joliot made a discovery of the utmost importance. He found that aluminium and other light-atom substances were rendered radioactive by bombarding them with alpha-particles. He showed that these radioactive products emitted positive or negative electrons and gamma-rays. By chemical methods he proved that the radioactivity produced was due to the formation of unstable isotopes of neighbouring elements, for example, in the case of aluminium, the active substance produced was an isotope of phosphorus. It was soon shown that the production of these artificially radioactive isotopes was possible by bombardment with other types of particle and that practically all the elements could be prepared in radioactive form. In this way Joliot founded an important development of modern 'tracer' technique and opened up a field of investigation of immense importance to physical, chemical, and biological science.

In 1939 Joliot and his co-workers observed that in the process of fission of uranium several neutrons are emitted. This important observation indicated the possibility of a chain process in uranium and Joliot at once endeavoured to produce such a self-propagating reaction. With Halban and Kowarski he showed, in 1940,

that if the fission neutrons were slowed down in heavy water a chain-reacting system could be produced. The practical success of the heavy-water 'piles' in the United States of America and in Canada is the outcome of these pioneering discoveries in the application of atomic energy.

In a great part of his work, Professor Joliot has been closely associated with his wife, Professor Irène Joliot-Curie.

I am grateful to several members of Council for their invaluable help in the preparation of the notes on the Medallists.

On 30 January Her Royal Highness Princess Elizabeth was elected into the Fellowship and she graciously attended at the Society's Rooms for formal admission on 3 July.

The Society is a privileged body and on the auspicious and happy occasion of the marriage of Her Royal Highness Princess Elizabeth to Lieutenant Philip Mountbatten, R.N., an Address was presented to His Majesty the King. The President, Vice-Presidents and Officers attended for this purpose at Buckingham Palace by Command on 6 November. His Majesty the King replied as follows.

'It is with special pleasure that I have received from the Royal Society, with which the Crown and my family have been so intimately associated for so many years, your address of congratulation on the occasion of the engagement of my dear daughter, who was so recently elected into your Fellowship On behalf of the Queen and myself, I thank you sincerely for the warmth of your congratulations and for your good wishes for the future happiness and prosperity of Princess Elizabeth and Lieutenant Mountbatten.   George R.'

Fellows will join me in expressing sympathy with Sir Alfred Egerton He has borne the pain and tedium caused by his accident with the greatest fortitude and cheerfulness and we hope that he is now well on the way to complete recovery of all degrees of freedom.

At the last Anniversary Dinner I referred to the relinquishment of the Foreign Secretaryship by Professor A. V. Hill and added, 'It is a comfort to know that he will not be out of reach. I am sure that we shall need his help and advice in the future and am quite as sure that it will always be willingly given'. A few weeks later it was most unfortunately necessary to find a temporary substitute for Sir Alfred Egerton and very fortunately the truth of the remark I have quoted was quickly proved. We are indeed grateful to Professor Hill for stepping into the breach as Acting Physical Secretary during the first six months of the year.

The Society was fortunate in securing the services of Dr D. C Martin as Assistant Secretary from the beginning of the year. By herculean efforts he managed to cope successfully with the operation of taking-over and also with continuing help in the organization of the Centenary Celebrations of the Chemical Society.

Undoubtedly the most interesting development in the relations of science and administration during the past year was the setting up of two Advisory Committees to the Cabinet The Defence Research Policy Committee was formed towards the end of 1946 and the Advisory Council on Scientific Policy at the beginning of the present year.

This represents the success in peace-time of an insistent demand of the war years for scientific consultation at the highest levels. Yet the consummation has evoked some uneasiness.

On the one hand it is feared that the freedom of research may be infringed by some form of planning or control Apart from the subtle effects of selective encouragement, a danger which can hardly be avoided, I do not share these apprehensions. As we might have expected, a very sound doctrine was clearly expressed by Sir Stafford Cripps when he spoke to us on the last Anniversary Day. His reasoned conclusion was that 'the real encouragement of pure research must be left to independent bodies like the Royal Society of London or the Universities'. There is every reason to believe that His Majesty's Government as a whole realizes the vital importance of untrammelled scientific research and will not interfere with its free development.

On the other hand it may be thought that the close contacts and collaboration with Ministers and their Departments which the Royal Society has enjoyed in the past are now endangered by the appearance of a buffer. There are several good reasons why no anxiety need be felt on this score and the chief is the enormous extension of public and governmental interest in scientific effort of all kinds. There is very much more work to do than we could possibly undertake alone, and a permanent organization with a full-time staff has become a necessity. The measure of our responsibilities continually increases and is likely to increase in the future. We have not surrendered the power to take the initiative, and we retain the traditional privilege of access to authority Our public usefulness depends therefore on our own ability to suggest and promote action and we have even gained potential for independent leadership Examples of our initiative during the past year are placed on record in the Report of Council and I draw your attention to three topics.

At the invitation of the Ministry of Education the Society has formed a British Committee for co-operation with U N E S.C.O in the Natural Sciences. This is perhaps the most widely representative scientific committee in the country. Its Chairman is the Foreign Secretary who is at present attending the Second General Conference of U.N.E S C O. in Mexico City as a delegate of the United Kingdom.

You will see from the Report that an investigation has disclosed that a major cause of delays in scientific publication is the shortage of skilled compositors. The Minister of Labour and National Service expressed interest in our problems and we are deeply indebted to Mr Isaacs for his personal intervention and for his suggestions. As a result of these and contacts with officials of the Printers' Trade Unions it is hoped that the situation may improve.

The following figures show the position of our own publications:

DELAYS IN PUBLICATION

Comparison of the time elapsing between the receipt and publication of papers in the years 1938, 1946 and 1947 (to September).

Journal	year	average delay (weeks)	maximum delay (weeks)	minimum delay (weeks)
Proceedings A	1938	18·9	25	12
	1946	57	68	42
	1947	35·5	41	29
Proceedings B	1938	16	23	10
	1946	65	88	33
	1947	41	53	32
Transactions A	1938	36·5	56	17
	1946	72	84	60*
	1947	55	66	38
Transactions B	1938	28·5	38	20
	1946	106	216	42
	1947	85·5	116	45

* Not including papers delayed by security.

The delay is roughly twice as long as it should be on the assumption that the practice in 1938 was reasonably expeditious. It is to be feared that the publication lag in many scientific journals is still greater and the harm that results is hard to overestimate.

It is discouraging to the younger men whose advancement often depends on a record of publications, it allows of anticipations by other workers, and hence to a proliferation of preliminary notes.

The third topic is that of the hoped-for improved accommodation of the Scientific Societies, it has been adequately discussed in general terms on many occasions We have continued talks and negotiations in various quarters during the past year But it became evident that no agreed scheme of co-operation between the Societies most nearly concerned had been prepared and that we have no precise case to advance. In order to provide this pre-requisite for action, a committee or working party has been formed by the Council to explore the problem in detail and to prepare the alternative complete plans

Before proceeding to discuss some new scientific matter I would like to read a greeting sent by Council on behalf of the Society to Sir Charles Sherrington on the occasion of his ninetieth birthday.

'The Royal Society sends you its greetings and good wishes for your birthday We think of our former President with especial pride and affection—we are proud of your scientific achievement, of the fresh light you have thrown on the hidden dealings of the nervous system, of the many pupils you have inspired and of the originality and distinction which has informed your scientific writing. We are proud of the philosopher who has first learnt the wisdom of the body and of the

poet who can touch the springs of the mind. But on this birthday we think not so much of the great scientist as of the well-loved friend of many years who has won universal esteem by his kindness and generosity and by the courage and purpose of his life. You have set us an example for which we thank you, and we wish you all the happiness which you have so well deserved.'

AN ESSAY IN CORRELATION ARISING FROM ALKALOID CHEMISTRY

The incident which I wish to describe is of peculiar interest because it transpires that the behaviour of a natural product can only be explained in the light of knowledge comparatively recently acquired and never previously brought to bear in the analysis of a structural problem

When we break up molecules in order to examine the fragments, or submit them to transformations in order to recognize the reactive groups, the changes that occur usually follow relatively simple rules; the pieces, for example, are clearly related to the whole from which they were obtained. In certain classical instances the picture has been confused by rearrangements of atoms within the molecule in the course of some of the changes examined.

But these molecular shifts also follow laws which have gradually been brought to light and it has always been possible to integrate the data in terms of a particular molecular structure.

Thus camphor showed itself to be a chemical proteus and at one stage it was not clear which manifestation corresponded to the reality. The vagaries of this molecule which proved most puzzling were explained by Lapworth. Camphor and all its derivatives have been synthesized and thus all dubiety was resolved.

Brazilin is another molecule which twists itself into contortions and here again we can follow all the varied transformations and we have the confirmation of synthesis, not it is true of brazilin itself, but of many of its more important derivatives.

But the star performers in the team of molecular acrobats are undoubtedly the alkaloids of the morphine group and I shall speak especially of thebaine.

In 1923–5 I discussed the chemistry of morphine, codeine and thebaine with John Masson Gulland, whose untimely death in a recent railway accident was so great a tragedy. We arrived at a solution of the problem which has since been generally accepted but there is no synthetic proof that applies to the thebaine structure itself. The certain knowledge from analysis and synthesis relates to the various transformation products and the molecular structures of many of these are established beyond doubt. The validity of our formulae, therefore, is estimated entirely on their success in interpretation of a complex of data. With an emendation in one group of products, suggested by Schöpf and gladly accepted, it seemed that the book was all but closed. Yet the most remarkable rearrangement of the molecule and one of the most extraordinary changes yet encountered in the whole domain of organic chemistry, remained to be recognized. It subjected the Gulland-

Robinson formula to a very severe test and triumphant emergence from the ordeal was at one time in doubt and has only been guaranteed during the last two months.

In 1905 Freund treated thebaine with phenylmagnesium bromide and obtained a new base, phenyldihydrothebaine, which was just thebaine to which the elements of benzene (C_6H_6, H) had been added. Similar substances were made later using other organo-metallic reagents and Lyndon Small and his collaborators have carefully re-examined and extended the series They have established some intriguing stereochemical relationships and corrected mistakes of other workers.

In 1943, in the course of a visit to Washington, I was privileged to hear of Dr Small's results and to examine the detail of his brilliant experimental work. Two conclusions appeared to me then to be inescapable but the full solution of the mystery remained elusive. In September of this year Dr Small kindly sent me the manuscript of a paper which should appear in the *Journal of Organic Chemistry* for November. He had been unable to make any use of my views and further correspondence showed why that was so, and also led to the understanding that the present disclosure should be made. A new idea, based on the postulates of 1943, then occurred and the validity of this has recently been indicated by a crucial experiment.

The first of the inescapable conclusions to which I have referred is derived from a comparison of thebaine with phenyldihydrothebaine The latter is outstandingly more stable. It is hard to hydrogenate in the presence of a catalyst (thebaine is easily attacked) and when reduction does occur all that happens is a break of a C—N bond Thebaine is very easily hydrolyzed as an enol methyl ether but although phenyldihydrothebaine contains this methoxy group (also one other OCH_3 and a phenolic OH), it is not hydrolyzed by hot concentrated hydrochloric acid. But hot hydrobromic acid produces the change $(OCH_3)_2(OH) \rightarrow (OH)_3$ and the action of diazomethane on the trihydroxy-compound gives a trimethoxy-compound which is the methyl ether of phenyldihydrothebaine. From these facts it is perfectly clear that the near-aromatic nucleus of thebaine, which bears the hydrolyzable methoxyl, has become truly aromatic in phenyldihydrothebaine.

The second conclusion follows logically from the first, considered in the light of other data. Phenyldihydrothebaine is optically active and by Hofmann's exhaustive methylation procedure it gives a series of optically active derivatives; this is even true of the second stage in which nitrogen is eliminated. The process is C—C—N—C—C → C=C ⋮ NMe_3 ⋮ C=C. The two double bonds in this optically active product can easily be shown to be present by diagnostic tests.

Now phenyldihydrothebaine is a C_{25} base and one carbon atom is in NCH_3 (now lost), two carbon atoms are in OCH_3 groups, and by hypothesis eighteen carbon atoms are in three benzene rings (two of the thebaine skeleton and the phenyl group introduced by the reagent); that leaves only four carbon atoms for the two double bonds. Hence exhaustively methylated phenyldihydrothebaine cannot contain an asymmetric carbon atom and it must owe its optical activity to

an *asymmetric molecule* of the type familiar to synthetic chemists and stereochemists but never before encountered in the study of a natural product.

It is easy to guess what kind of an asymmetric molecule is present. Thebaine is easily degraded to phenanthrene derivatives (such as I) and these in their turn are derived from diphenyl (II).

(I)   (II)   (III)
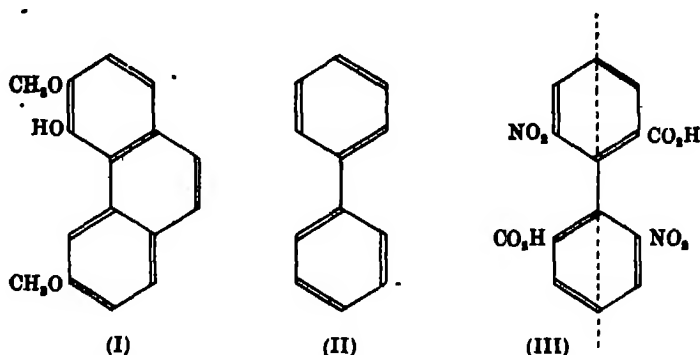
In 1922 Christie & Kenner showed that the dinitrodiphenic acid (III) could be resolved into optically active forms. They also pointed out that this result was explicable if the two rings are assumed to be co-axial (as shown by the dotted line) but not co-planar. In 1926 Turner & Le Fèvre, Bell & Kenyon, and Mills developed the hypothesis that restricted free rotation of the phenyl groups is conditioned by the position and size of the substituents It is well known that this hindrance theory harmonizes all the extensive experimental data

We arrived at the restricted diphenyl idea as a plausible explanation of the optical activity of the end-product of the Hofmann degradation but there is strong evidence that it must also be applied to phenyldihydrothebaine itself

Small and his collaborators have shown that Freund's phenyldihydrothebaine is a mixture of two isomerides (equated in various ways, as by reduction, or exhaustive methylation) which he calls $(+)\alpha$ and $(+)\delta$. On heating $(+)\alpha$ is partly changed to the optical antipode of $(+)\delta$, similarly $(+)\delta$ goes partly into the optical antipode of $(+)\alpha$. The equilibria can be reached from either side. Small clearly recognizes that these changes are partial racemizations but appears to think it possible that a number of asymmetric centres could be inverted in step. He calls the optical antipode of $(+)\alpha$, $(-)\alpha$, but it is preferable to term it $(-)\delta$. Thus $+$, $-$ and α, δ can be taken to be symbols representing left-hand or right-hand at each of two sources of dissymmetry. The partial racemization of $(+)\alpha$ gives then $(-)\alpha$ These facts alone suggest that the phenyldihydrothebaine molecule contains two sources of dissymmetry and only two, one of these (represented by $+$, $-$) is a noncoplanar diphenyl configuration, and the other (represented by α, δ) is an asymmetric carbon atom. We are able to substantiate this view and the substance is therefore the first in which molecular dissymmetry and

that due to an asymmetric carbon atom (also, strictly speaking, molecular) co-exist. The theory extends to this case without modification; there are the usual numbers of isomerides, 2^n, where n is the number of sources of dissymmetry, not necessarily all asymmetric carbon atoms. This principle is quite obvious but has not previously been enunciated, chiefly because the mixture of asymmetric types is itself new.

All these and many other facts had to be taken into consideration in the endeavour to find a structure for phenyldihydrothebaine and it would take too long to carry the argument through all its stages. It will be stated more fully elsewhere; meanwhile phenyldihydrothebaine is believed to be (V) and its formation from thebaine (IV) is the result of molecular rearrangement of quite a new type.

(IV)   (V)
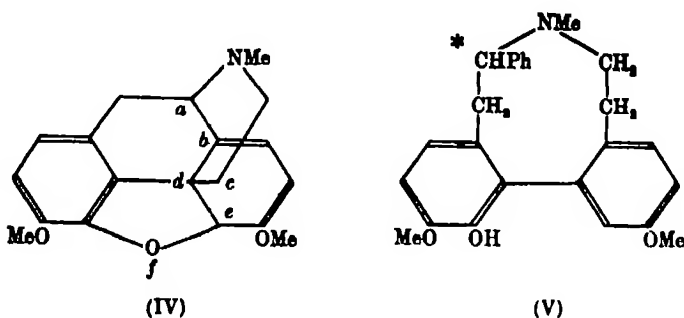
It was first formulated as a likely process from the electronic point of view. In words the migrating ethanamine chain is cationoid, (*c*) is joined to (*b*) by means of the electrons of the link (*a b*); the electrons in (*c d*) go to (*d e*) and fulfil the demands of aromaticity of the nucleus, electrons (*e f*) are then taken by the oxygen atom which acquires a negative charge (on decomposition of the complex by water, a proton is attached to oxygen): the phenyl anion brings a new electron pair to (*a*), replacing those lost to the migrating group (*c*). This connected series of changes can be thought of as starting at either end, i.e. attack by Ph—, or assumption of a negative charge by oxygen. As in many other migrations in this group the driving force is doubtless the tendency of the near-aromatic group to become fully aromatic. The formation of isomerides is due to construction of the —CHPh— group in each of the two possible senses, the diphenyl skeletons being configured in one sense only.

The formula (V) is perfectly satisfactory, examination of models shows that the nine-membered ring allows the phenyl nuclei to be disposed at right angles or at any intermediate angle without strain, there is one asymmetric carbon atom marked * in the formula (V); all the chemical properties and transformations receive natural explanations. But there was thought to be one important exception and because of it Dr Small was not disposed (some time before the complete theory was available) to accept the postulation of the two aromatic nuclei. The ultra-violet

absorption of phenyldihydrothebaine gave no indication whatever of the appearance of a new aromatic ring in conjugation with the first.

This ought to have been thought decisive, but as it conflicted with other equally definite evidence, something had to be jettisoned. Actually a further correlation is possible and the parts now fit together like a Chinese puzzle made in ivory.

I am indebted to Dr J. W. Cornforth for pointing out that non-coplanar diphenyls do *not* exhibit normal ultra-violet spectra. This was first noted by Picket, Walter & France (1936) and a clear case is given by O'Shaughnessy & Rodebush (1940). In this example a large ring of eighteen members is formed by a bridge across the *m*-positions of a diphenyl derivative. There is no diphenyl band and only end-absorption.

In the last few weeks we have sought and obtained experimental confirmation of the structure (V)   Mr K. W. Bentley has oxidized phenyldihydrothebaine with potassium permanganate and obtained benzoic acid, benzaldehyde, and 4-methoxyphthalic acid (VI). This was identified very thoroughly with an authentic specimen

By exhaustive methylation, including that of the phenolic group, an optically active product (VII) (made by Freund but stated to be optically inactive) was obtained and oxidation of this by permanganate in acetone afforded a trimethoxy-diphenic acid (VIII). These are very cold, hard facts and it is to be feared that in the future they will be cited first and all our interesting arguments will be forgotten.

(VI)   (VII)   (VIII)
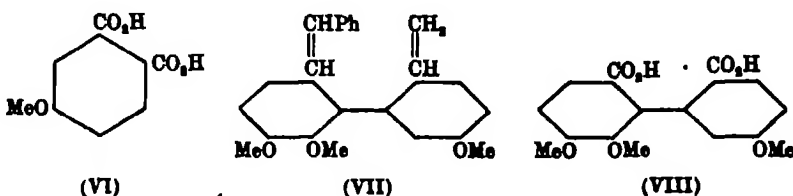
One further punctilio   The thebaine transformations are like the variations of a chess problem in that they have a common key-move. Are we justified in assuming that the problem as set has a unique relation to the variations? Is the thebaine structure proposed by Gulland & Robinson the only one which serves to explain the whole of the results? The key-move is the migration of the ethanamine chain from (*d*) to (*b*) in (IV); it is only the position *after* the migration that we have proved to be correct. Taking everything into consideration one alternative can indeed be seen. This can best be explained by reference to the intermediate (IX) now postulated in the complex changes that thebaine undergoes in acid solution.

(IX) arises from (IV) by migration from (*b*) to (*d*) and by hydrolysis. It could equally well arise from (X). The only way of distinguishing between (IV) and (X) seems to be by a study of the α- and β-methylmorphimethines and the optical data on these substances strongly support (IV) and would remain inexplicable on the

basis of (X). According to Dr R. Strauss the-ultra-violet absorption of α-methyl-morphimethine is not styrenoid and would be consistent with the arrangement, $\phi.C.C:C.C:C$—. On the other hand that of the β-isomeride shows strong conjugation consistent with $\phi.C:C.C:C.$. The skeleton of (IV) permits these rearrangements and structural elements, but that of (X) does not. The question is related to that of biogenesis and further enquiries must be instituted.
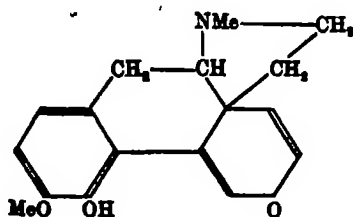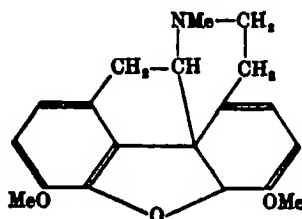
(IX)   (X)

The Research Laboratories of the General Electric Company

By Sir Clifford C. Paterson, F.R.S., *Director of the Laboratories*

[Plate 1]

The lecturer introduced his subject by showing lantern slides illustrating the buildings of the laboratories, also certain processes and tests in progress in various departments

I am glad of this opportunity to indicate the objectives and the *modus operandi* of the laboratories of which I have been Director since their foundation in 1919. Such a study may raise, I hope, in the minds of some of us, the question of what should be the objectives of research when attached to productive industry— remembering that the rate at which efficient and ordered manufacturing industry can digest new diets of innovations and novel products is inevitably limited, much more limited I think than is usually appreciated by those who are not themselves engaged in productive industry.

The staff engaged at these laboratories now totals rather over 1000. As the establishment is entirely self-contained, that number has to include all essential service personnel in addition to the professional scientific and engineering staffs These latter number about 250. Physicists and engineers predominate in numbers over the chemists, metallurgists and glass technologists. There are also some 100 young men who are going through their professional training with us in science and in research, and working for B.Sc. degrees or Higher National Certificate qualifications on a part-time basis. Whilst they are passing through this stage they constitute our junior technical staff, to merge in time (or most of them) into the fully qualified research staff There are, among the total number, 160 skilled craftsmen of all trades who are as much 'on the staff' (with equal staff status) as the professional personnel. We started in 1919 with a staff of about thirty and have grown to these numbers at a fairly even rate over 27 years.

Objectives of the Research Laboratories

Now as to our objects. I would like to dwell upon these, for it was the subject of no little discussion at the start, and I am glad to say there was then no difference of opinion, nor has there been since.

The laboratories have had three main functions.

(*a*) The first and most important is to give scientific service to the factories and departments of the Company.

(*b*) Secondly, from the intimate experience so gained with these factories and departments, to draw inspiration to formulate and carry out such longer-term

researches as in the opinion of the laboratory staff will ultimately benefit their industry.

(c) Thirdly, to join in any outside activities in which an industrial scientific staff can help the professional life of the community.

The essential fact I would like you to note is that (i) the starting-point for the grouping of the research work within the laboratories, (ii) the number of men devoted to any field of work, and (iii) especially the organizational link with factories and departments of the company we serve, are based upon the requirement of giving them scientific service in all the ways in which an up-to-date laboratory can help them.

One of these ways (covered by the second objective) is of course to watch all trends and prosecute a certain number of long-term researches which seem likely to lead to outstanding additions to knowledge or drastic changes in technology. This latter function is of course very important, but it is not in fact the primary one for which our laboratories exist.

I believe this is of interest because it seems to be a reversal of the more usual set up Under this an industrial research laboratory is designed to keep all its thoughts and intentions upon investigations which will lead to new processes and products. The giving of scientific service to existing industry is hardly part of the design· it becomes an incidental duty which is regarded as tending to interfere with its principal and more fundamental functions.

At first sight this difference of objective may appear doctrinaire, but in fact it vitally affects the structure and outlook of the scientific side of the electrical industry so far as my Company's part of it is concerned. I think I can also establish that the scientific approach to an industry, through an intimate knowledge of its existing products and processes, forms the most effective of all vantage points from which to get inspiration for the right sort of long-term as well as short-term researches. There are always so many investigations which can be attempted—so many temptations to unnecessary and ineffective experiment—that the choice of what to do can be a difficult matter unless dictated by first-hand intimate knowledge of the manufacture in question, in all its aspects.

In the days when it was necessary to commend to industrialists the importance of scientific research, I used to reckon that the expensive things they were persuaded not to undertake, went a long way to meeting the cost of my establishment. That is I know a common experience with many of us, but we sometimes forget it in assessing our contribution to industry.

Before I explain by examples how this works out I must just indicate the scope of the factory production units and the like which we exist to serve.

THE GENERAL ELECTRIC COMPANY

The total personnel in the General Electric Company is about 50,000. It was about half this size when we started, but its main units already existed then. Its motto has been 'everything electrical' so you will expect a wide scope of effort.

The factories we were to serve were autonomous establishments widely separated geographically:

A steam turbine and heavy mechanical engineering works at Erith

An electric cable works at Southampton.

Electrical Engineering Works for heavy rotating machinery, transformers, switchgear and rectifiers, as well as light electrical engineering plant, at Witton, Birmingham.

Electric lamps, radio valves and glass, London.

Measuring instruments and apparatus at Salford and Birmingham.

Telephone and radio works at Coventry.

Electro-medical works in London.

Electrical appliances (household and industrial) at Birmingham.

Carbon and battery works, Birmingham.

Central Sales Department in London with Sales Branches in most large cities

A number of smaller factories for subsidiary products

It might have been a baffling matter in 1919 to know how to begin, had it not been for the definite principle of approach which I have indicated plus the urgent request from the management to concentrate attention first on electric lamps and their utilization

FACTORY COLLABORATION

So you see us starting as a group of industrially raw but enthusiastic scientists approaching a factory staff who had long experience and were justly proud of their production skills and achievements. This was an inflammable situation in which success depended as much on a study of human nature as upon grasping the technical nature of the problems and upon learning the processes

The problem of welding two such staffs into what is in effect a single team is in my view the essence of successful industrial research and does much to determine the form of the internal organization of the laboratory. It is a battle which is never finally won and the price of its continuance is eternal vigilance.

I have dwelt on this because the same battle has to be fought in every factory with which we collaborate Almost invariably it falls to the lot of the laboratories to lay siege to the good-will of the factory production staffs at all levels. It is indeed an essential and most beneficial part of the training of the laboratory staff to do so— and ultimately to learn that it must not be one of their objectives to show the factory managements how clever their research people are.

STAFF STRUCTURE

So, in the internal structure of the Laboratories there must be a distinct group of workers with a group leader for each factory or each important section of a factory (or technical Department) of the Company. There are thirty-four of such laboratory groups, the membership of which varies from say three to twenty

qualified people each. With us the individuals in the groups are mostly physicists, engineers and chemists.

We have in addition the following strong service groups which are at everyone's disposition. analytical chemists, metallurgists, X-ray workers and spectroscopists, mechanical engineers, glass technologists, and statisticians.

These, who constitute about 10 % of the scientific staff, are at the service of any of the groups working with the factories, and join in their specific researches as required.

The whole set-up is flexible and somewhat informal—partly because there is no apostolic succession of conferred authority or status or staff grades of seniority Some thirty of the most respected members of the scientific staff are designated Leading Staff. That is the only discrimination in staff status. But the leadership referred to in this case is intellectual and also a matter of character. It is not administrative Whilst therefore it carries its inevitable moral responsibility it bears no conferred authority. Group Leaders are not necessarily members of the Leading Staff—nor are members of the Leading Staff necessarily Group Leaders

I must not pursue further the subject of internal staff organization—which, however, I myself find of absorbing interest. I only bring it into this description of the place in order to indicate how we manage to have some thirty-four separate groups nominally responsible to the Director.

The guidance of the work of any group under its group leader is much assisted by the attendance of its members at regular meetings with their opposite numbers from the factory (or technical department) which the group serves At these meetings the status of products and processes is discussed from the technical and marketing standpoints These meetings are interlocking and advisory in their character and have no authority actually to direct development or research.

This structure of interlocking meetings was a matter of gradual growth. It derives its standing from the recognition and support of the Chairman and Managing Directors of the Company—an important proviso.

<div align="center">PUBLICATION</div>

From the beginning, publication of the scientific aspects of the work of the laboratories has been pressed. Papers are nominally communications from the scientific staff as a whole, under the authorship of the individuals who have been mainly responsible for the experimental work. The technical aspects of the work are also published where they are likely to be of general interest, but the publication of the technical 'know-how' of processes is as a rule reserved.

It follows that, if a competitor has a well-staffed research establishment of his own, he will be able to derive assistance from studying our scientific publications, and we his; but if he is one of those who merely seeks to trade on other people's

work, by cadging information so that he can set up manufacture on empirical lines but making no contribution to knowledge himself, we try not to help him.

There have so far been some 100 major publications to the learned societies, and there is no impediment to joint authorship with staff from outside research establishments.

The policy lying behind this is not merely a philanthropic one. It brings of course its own rewards, one of which is that no research staff can be healthy if it works under a ban on reasonable publication

TRY-OUT UNITS

As the practical outcome of much of the work must show itself in better or cheaper products for the market, the test of every new development to this end ultimately lies in the making of something   It is necessary therefore for some of the groups to have nucleus production facilities with personnel whom they have trained in the particular skills required (after learning them themselves)   These units are located within the laboratories in close proximity to those research workers who guide them and use them.

It has sometimes been a hard battle to persuade a factory production staff that amateurs (particularly scientific ones) can make any useful contributions by trying to produce articles themselves. I think this battle was finally won during the war. In 1940 it was obvious that there was no one ready or able to make the many types of radio valve developed for centimetric radar and the like   So we greatly intensified our amateur efforts. We called our little groups pre-production units and expanded them to the utmost till the number of their personnel reached 700   There were many tears and much perspiration, but we mastered the production problems of each new type of valve as it emerged from the research stage, and by the end of the war had made in the laboratory 360,000 valves of forty-eight types at a cost of $1\frac{1}{2}$ million pounds.

I think the factor which is often overlooked is the very great educative influence which the effort required actually to produce articles has on a scientific staff. The mere making of an article or demonstration of a process can be a relatively small matter compared with putting it into efficient and uniform production whether by unskilled, and maybe unsympathetic, human effort or by skilled and complicated mechanical agencies.

In industry uniformity is a gauge of quality, but the achievement of uniformity presents major problems where every operation has a Gaussian distribution of variability, the causes of which must be found and defined, and where hundreds of such operations and components go to the make-up of the product. Herein lies one of the largest single sources of the research problems which force themselves upon the attention of such a laboratory as ours.

As you will imagine, the solution of many of them requires prolonged research, using the best available resources in the way of equipment. They constantly lead to most interesting investigations, which I hope I may now exemplify.

Example 1. Glass and refractories

Glass

Take, as my first example, the field of glass. We first met glass in the making of bulbs for electric lamps. These bulbs are delivered from the glass factory to the lamp factory as open ended envelopes which are then assembled with the other components on somewhat intricate machines These melt or soften the glass at the right points by means of gas flames. Each bulb follows the previous one through the automatic machine at the shortest possible time interval needed for the melting of the glass and its manipulation There is no chance of hand control. The timing of the operational sequences of the machine is remorselessly uniform. Thus the amount of heat applied, and the behaviour of the glass under the heat must be very uniform indeed or chaos results.

By skill in empirical operation the factory were managing to use as few as 125 bulbs for 100 good lamps Was there anything we as research men ought to do to reduce the wastage? We undertook of course a comprehensive study of the influence of the various constituents of the glass on its viscosity over the required temperature range. It was a long road involving continuous work and study in the glass factory. In the end we reached an efficiency of 104 bulbs for 100 good lamps

The softening point is only one factor in glass quality The coefficient of expansion is another—ensuring that it shall seal on to other glasses without strain or with the designed amount of strain either in tension or compression Then there are the wetting properties when sealing to metals and alloys—also the electrical conductivity where the design of the lamp requires that parts of the glass must operate at relatively high temperatures. Over the past 25 years the study of these things has led to the design of glasses for electronic devices which respond to very exacting demands, and without which the devices would not be practicable

The composition of glasses may involve eight constituents, each having its influence upon the characteristics.

Refractories

It is well known, however, that the art of making good glass is wrapped up with the art of making good refractories in which to melt the glass: molten glass is chemically one of the most active materials—it will attack most things. Therefore, if uniform glass of known characteristics is needed, a grasp of the science and art of refractory manufacture is essential.

· The problems of refractories are always with us for they are stimulated by the constant urge to produce special glasses, which are uniform and not subject to disastrous wastage May I give one example from the work of my colleague Dr Partridge?

By X-ray and other studies we are finding that fire-clay refractory materials consist of crystals of mullite and silica set in a glassy matrix It would seem clear that their properties at high temperatures must be influenced by the viscosity of this glassy cement Indeed we find that the behaviour of all kinds of refractory

materials at high temperatures can be understood more readily by considering them from this point of view.

Figure 1, plate 1, shows a large fire-clay crucible run at a temperature of over 1400° C. It collapses like this through weakness after a short life under the fluid pressure of the molten glass inside. Curve A in figure 2 shows the result of high temperature creep tests on this refractory and confirms its weakness.
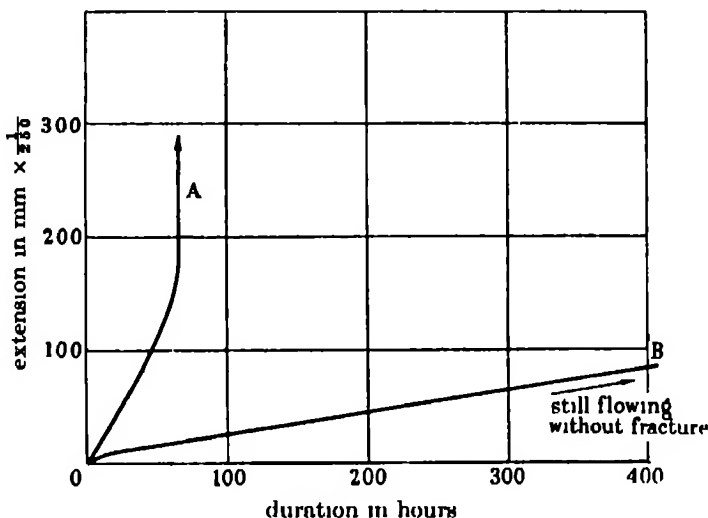
FIGURE 2. High temperatures creep tests of pot clays.

We have found that the eutectic mixture (i e the mixture of lowest melting-point) in the silica-alumina system is actually a glass This is significant because this eutectic mixture is present in all fire-clay refractories

When pure it is a very good glass and was the basis of a special glass we developed during the war for seals in silica valves, its melting-point was about 2000° C

Unfortunately other oxides present in the fire-clay, such as alkalis, iron oxide and magnesia, combine with this matrix glass to render it more fusible and mobile. Guided by this a search for other clays has yielded a refractory whose creep characteristics are represented by curve B of figure 2 and the pot which results is shown in figure 3, plate 1, after a long life Incidentally, the average life of the pots is now increased three times

Following the same line of investigation, figure 4, plate 1, shows a photograph of the inside wall of a glass tank which has been operating for many months with 80 tons of molten glass. A tank costs thousands of pounds to put out of action and rebuild. You can see the general corrosion which has been going on. Observe especially the row of white blocks about 12 in. high on the left side of the picture Refractory blocks of this shape originally extended along to the right-hand side— but here they have been simply eaten away and have gone into the glass and contaminated it. The blocks on the left were made from the new purer refractory,

which contained less of the glassy phase. These have now been generally adopted and the tank life has been lengthened greatly as a result.

It is not practical politics to make well-intentioned guess-work changes in the design and materials of these great tanks. The loss of too much money is involved in a bad shot Anyway, if anyone makes a mistake it must not be the scientist. Therefore all the resources of the research laboratory must be invoked to establish the soundness of a proposed change before making it, and the technical staff of the factory also have to be persuaded by our arguments. Investigations to this end usually take years before practical trial is made, and even then the results cannot be established for another two years or so.

Opal glass

Opal glass is used to diffuse the light from electric lamps and to obscure the intensely bright filament. The opacity was an empirical matter resulting from the precipitation of some constituent from clear glass as it cooled from the molten state.

Measurements showed that the diffusing power and the loss of light by absorption varied greatly in different opal glasses. There was much argument about the nature of the precipitated particles which caused the scattering of the light X-ray powder photographs showed us that they were, in fact, minute particles of calcium or sodium fluoride, or both. The particles are 'normally spherical but occasional brittleness of the glass was found to be associated with the development of sharp angular crystals. Another important result emerged from a mathematical investigation This showed that, consistent with satisfactory diffusion, the size of the particles was critical for low absorption.

These studies of the fundamentals enabled a more efficient opal glass to be made and, more important, enabled consistent results to be achieved in the glass works All that was 15 or 20 years ago.

One research of course often suggests another in some surprisingly different field. Thus, in 1940, when the practicability of centimetric radar was under active discussion, Ryde (remembering his earlier work on opal glasses) developed the analogous theory of the absorption and scattering of very short radio waves by rain and similar meteorological phenomena. This was followed by detailed numerical predictions of the attenuation and the magnitude of the radar echoes to be expected at different wave-lengths in the centimetre band. These provided much needed information at a critical time when actual measurements of the effects were out of the question. So much for my examples from glass.

Example 2. Filament lamps

One of the research objectives stimulated by intimacy with the processes of lamp manufacture was the improvement of the efficiency of the lamp as a light giver. This could be effected by many details of (*a*) tungsten wire processing, (*b*) filament coiling, (*c*) gas-filling and (*d*) glass making. The immediate incentive to improved

Paterson

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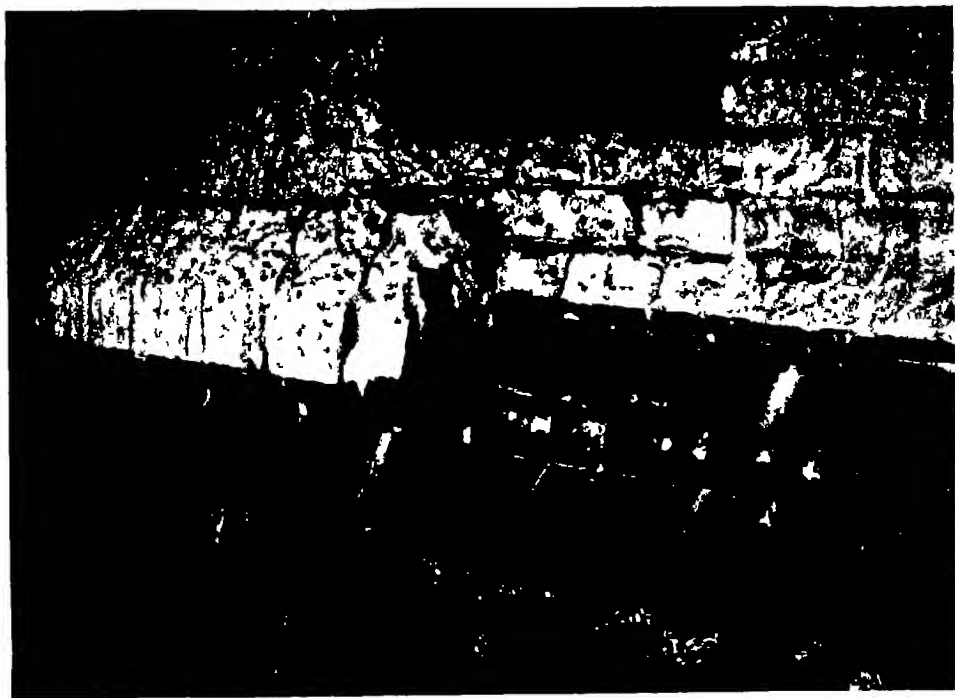
FIGURE 1

FIGURE 3

FIGURE 4

efficiency was the normal competition on the market for lamp quality and ability to show more economical use of the electricity for which the user paid.

It is interesting to look for a moment at what this means now from the national standpoint. Electric lighting in this country uses some 4000 million units of electricity per annum. Every 10 % of electricity saved in getting from the lamp the light we want, means in the end a saving of some quarter of a million tons of coal per annum, together with the incidental saving of generating plant.

Example 3. Discharge lamps

We worked for a number of years on the above lines during which the average filament lamp efficiency was increased some 25 % It was clear, however, that little further worth-while gain in efficiency of filament lamps was likely.

A study was therefore taken in hand by a special group (also under J. W. Ryde) of the possibility of the electric discharge in gases and vapours being made to yield useful light. This turned out to be a fortunate inspiration which resulted firstly in the high pressure mercury lamp for street lighting, then the mains voltage luminescent tubular lamp for internal lighting, besides various types of compact source high intensity lamps for projection As these lamps have now three times the efficiency of filament lamps, when they become generally adopted we shall be able to enjoy the same amount of light in our buildings for one-third the amount of electricity consumed. This was a really fine gain, of which the country has yet to reap the harvest It means a potential saving of nearly 2 million tons of coal per annum. In terms of electrical generating plant it can mean (assuming a 10 % lighting load factor) the saving of something like three large generating stations of one million kilowatts output each, or say five stations of the present size of Battersea.

These revolutions are but vaguely appreciated because they materialize over a number of years and also because of the natural inclination of the public to employ some, at least, of the advantages presented to them in having more light rather than in using less electricity

I have no time to mention any of the many interesting problems we met—and still meet—in this field of fluorescent lighting. I refer to it because of course it is an example of a complete break away from existing practice and takes its place in the second of the objectives I mentioned at the outset Nevertheless, I would insist that even a break-away product like this needs to be evolved from the beginning by people who have an intimate acquaintance with the appropriate manufacturing procedures and materials.

The thermionic valve

I was tempted to take some examples from the extensive field of the development of the radio valve, which has been another of our major subjects of scientific study and development ever since its industrial birth during the first world war. This product indeed rests on the giving of scientific service through intimate

association with its manufacturing arts—and affords a wealth of evidence of the theme I am trying to develop. But time and space will hardly permit of further examples of this kind

PROJECT RESEARCH

There are some categories of equipment manufacture which are assisted best when the associated research group bases its work on projects rather than on scientific service This applies, for instance, to telecommunications, both radio and line telephony Thus, the project for distributing television over the country by radio links on centimetric wave-lengths involves some interesting wide wave-band transmission and reception problems. Another is the use of radar techniques for injecting pulses into circuit networks; these pulses probe the electrical characteristics of the circuits and cause them, as it were, to write their own signatures in cathode-ray tube traces

Some of the war-time projects of this kind, which our groups undertook, made history, for in the first half of 1940 they produced the first centimetric radar and made the first practical cavity magnetrons, the principle of which had just been demonstrated by Randall and Boot. This was followed by the evolution of the radar project known as Oboe, with the leadership and co-operation of T.R E.

User or Operational Research is in an intermediate category It formed the basis, for instance, of our investigations into street lighting, where it was found that visibility on the road depended upon making the road as light as possible and objects upon it as dark as possible—a principle which resulted in a radical change in the design of luminaires for street lighting and their location on the roads

QUALITY APPRAISEMENT AND CONTROL

Whether research is based upon the giving of scientific service or upon the study of new projects, or whether some entire break away in product or process is in hand (wherever there is manufacture) there is always the problem of statistical appraisement of the quality of the manufactured product followed by the introduction of some system of quality control.

This has been proved, by the experience of ourselves and others, to be the key to efficient and intelligent production We find that it is best introduced to the factory at the craftsman (or charge hand) level (not at the managerial level) by scientific men who, as I have explained, are prepared to immerse themselves in the factory regime.

The practical principles of this quality control are simple—almost childish—but the psychology is often baffling. In our experience the secret of success is to work through the intelligent engineer in the factory and to instil into him the very small but essential amount of statistical technique. The ineffective way is usually to work through trained statisticians, and to expect them to assimilate sufficient knowledge of the processes concerned to ensure a sound practical result—unless of

course a statistician can be found who is also an engineer with a gift for immersing himself in factory problems and who has patience to concentrate upon the application of quite simple statistical procedures.

Factory men of the right enthusiasms and psychology are difficult to find Without the constant encouragement and guidance of the practical statisticians on our research staff, we find this powerful weapon of progress tends to rust and lose its effectiveness

CONCLUSION

I could touch upon other fields of the work, for instance, (a) hard stones like diamond and sapphire and their handling, (b) crystal growing, (c) powders and the techniques for handling them, (d) heating for buildings and for industrial processes, (e) household appliances, including cooking, (f) carbon, (g) insulating materials, (h) X-ray and other electro-medical equipment, (i) steam and gas turbines, (j) heavy electrical equipment They are very diverse, but the procedures for handling them follow closely the lines I have already sketched.

There is one group of activities which differs somewhat from these in character We put the resources of the laboratories at the disposal of Government Departments by accepting research or development contracts for investigations for which our particular skills are suited. Probably one-fifth of the research resources I mentioned earlier are occupied in this way.

As you see, I have not attempted in this talk to give a review of the work we are doing, but rather of the way our laboratories have learnt from their experience to go about the work. I have done this not merely in order to describe the establishment, but in the hope that you will allow me to point out a consideration of general importance, the factor of efficiency in the use of scientific facilities in industry.

I sometimes feel we have the idea that we have only to do research and get explanations and knowledge of certain things and the rest follows naturally. If we do not get explanations and knowledge of exactly the right things, and if we produce that knowledge in other than a favourable psychological atmosphere, things just do not happen, or only just happen, in the industry we are trying to serve, notwithstanding all our imposing and praiseworthy treatises and reports, some of which are good of course, but how many?

An extreme case may be that of a detached research laboratory which merely publishes its reports and leaves industry to do all the thinking about whether the results can be made of use for practical purposes How much of the whole amount of such effort really gets into an industry in a form in which it brings about advances which pay a technical dividend depends naturally upon the level which that industry has reached. But it is I fear, usually a small proportion, say 10 or perhaps 15 %. Even so, that is useful.

A stage higher in effectiveness may be the research establishment which, having done a piece of research, works hard to demonstrate the practicability of the results. What proportion of the total man-power effort of that establishment will

have been effective in producing a technical dividend in manufacturing industry? Shall we say 20 or perhaps 30 %?

I contend that when scientific manpower is being employed in the manner I have been trying to indicate in this talk, its efforts have as a rule a maximum of effectiveness in producing technical progress within industry. The stimulating reaction on most research workers of being in contact with the actual application of their work constitutes one aspect of this picture. Another is the progressing of the investigations from their early stages in co-operation with the actual individuals who must implement them and make them serviceable.

I feel we need to foster the conception of the effectiveness and non-effectiveness of industrial research according to the kind of chance and help we give it to fructify, and it is to this end that I commend to you some of the considerations I have been discussing I hope I have indicated some of the criteria which, as I see it, can give us a sense of values in regard to efficiency in the employment of scientific resources in production industry.

I do not want to hold up my laboratories as an example to be followed Nearly every research establishment in industry has a unique problem in organization But laboratories like those I have been describing do, I hope, serve to exemplify certain principles which are worth examination.

The Plymouth laboratory of the Marine Biological Association of the United Kingdom

By F. S. Russell, F R S., *Director of the Plymouth Laboratory*

[Plates 2 to 8]

In *The Times* of 31 March 1884, it was announced that a meeting would be held that day in the rooms of the Royal Society for founding a society having for its purpose 'the establishment and maintenance of a well-equipped laboratory at a suitable point on the English coast, similar to, if not quite so extensive as, Dr Dohrn's Zoological Station at Naples' (M B.A. 1887a) With Professor T. H. Huxley in the chair a gathering of distinguished gentlemen gave reasons why such a laboratory should be built. All stressed what its value would be from the purely scientific viewpoint, and all were agreed that both by fundamental research and by more direct investigations on our food fishes, knowledge of economic import would be gained. The last speaker, Mr George J. Romanes, said that there was one function of the proposed laboratory which had not received the attention it appeared to

deserve; he meant the investigation of invertebrate physiology. 'In the inverte-brate forms of life', he said, 'we saw life in its simplest shape, and in the shape which best admitted of observation and experiment, with the view of throwing light upon most of the great questions relating to the processes of life' (M B.A. 1887 *b*).

As a result of this meeting a corporate society, the Marine Biological Association of the United Kingdom, came into being It was decided that the laboratory should be built at Plymouth where a rich and varied fauna was available The building, which was opened on 30 June 1888 (M.B.A. 1888), is situated under the walls of Charles II's Citadel in a commanding position overlooking the waters of Plymouth Sound

The Association, which owed its inception largely to the initiative and energy of Sir E. Ray Lankester, received and still receives financial support from persons interested in the study of biology and the sea, who are the members of the Associa-tion In addition considerable financial backing was given by a number of Uni-versities and other public bodies, and notably the Fishmongers' Company From its beginning an annual grant was made by H M Treasury, and after the 1914–18 war this grant began to assume considerable proportions when it came under the control of the Development Commissioners *

In 1902 the Association was asked by H M Treasury to undertake the English share of the scientific investigations which formed part of the programme of the International Council for the Exploration of the Sea (M B A 1903). The English share of these international fisheries investigations was divided under two heads.

I. A survey of the trawling grounds and fisheries in the southern North Sea, together with scientific observations on migrations, feeding and rate of growth of the more important fish For this purpose the Association also ran a laboratory at Lowestoft.

II. A hydrographic and plankton survey of the western half of the English Channel

After the 1914–18 war the Lowestoft laboratory 'was re-established under the Ministry of Agriculture and Fisheries for the purpose of studying problems having a direct bearing on the commercial fisheries. At the same time a substantially increased grant was made to the Marine Biological Association for the maintenance of the Plymouth laboratory, so that researches of a more general or fundamental nature concerning life in the sea might be developed on a larger plan' (Allen & Harvey 1928) The years between the two great wars saw an increased staff at the Plymouth laboratory, and constant additions to the buildings and improvement of facilities for research of all kinds.

Over the period that the Association has been in existence it is possible to trace a sequence in the development of the work from the titles of the many papers in the Association's own *Journal* and its other publications (M B.A. 1928). It should be

* An interesting account of some of the early history of the Plymouth laboratory is given by G. P. Bidder (1943) in his obituary notice of Dr E. J. Allen.

remembered that in the earliest days practically nothing was known about the life histories and habits of even our commonest food fishes, and few carefully compiled accounts of the commercial fisheries were in existence   Since the avowed objects of the Association (M.B A. 1887b) were to establish one or more laboratories on the British coasts 'where accurate researches may be carried on leading to the improvement of zoological and botanical science, and to an increase of our knowledge as regards the food, life, conditions and habits of British food fishes, and molluscs in particular, and the animal and vegetable resources of the sea in general', it was only natural that the emphasis in the beginning had to be on fishes. Accordingly, we find that a large number of the earlier papers were devoted to observations on the habits of fishes, shellfish and other products from the sea, and to the collection of information on the methods and results of commercial fishing. This was very necessary, because the few naturalists concerned had to have a general picture of these matters to be able to answer with some feeling of authority the many questions that must have been put to them once the Association was founded. I have no doubt that quick results were expected in those days as they still are in certain quarters to-day.

But in addition to doing fishery research, the few naturalists of the staff, together with a number of enthusiastic visiting workers, were building up a knowledge of the marine fauna and flora off Plymouth and its neighbouring coasts This again was a first requirement, because in all branches of science the systematic observations must come first. More direct fishery research also naturally received considerable attention as a result of the assumption of the English share of the investigations under the International Council for the Exploration of the Sea But some of the programme of these investigations gave opportunities for increasing the pure scientific observations and studying the environmental factors in the sea over a wide area. Thus much of the foundation was laid for our knowledge of the hydrological conditions round the British Isles, of the fauna of the sea floor, and of the microscopic plants and animals which drift with the water and form the plankton.

Looking back over the first twenty years of the Association's life it is remarkable how much ground was covered by the few research workers available and how well balanced on the whole had been the distribution of the investigations. A most valuable fauna list (M.B.A 1904, 1931) had been produced, a beginning had been made in the study of the distribution of the animals in relation to their environment, and fishery research had been put on a solid basis. Many papers on the morphology and development of marine organisms had been published, and, in addition, here and there appeared a paper somewhat before its time which gave advanced indications of other possible uses to which the Plymouth laboratory might be put.

After the 1914–18 war there was a noticeable change in the subject matter of the contents of the Association's *Journal*. With the taking over of fisheries research by the Ministry of Agriculture and Fisheries the work at Plymouth became for the greater part fundamental in nature. All the emphasis was on the study of the chemical and physical conditions of the environment, the life histories and develop-

ment of marine invertebrate animals, and the distribution in space and time of the animal populations. But at the same time the economic aims were not entirely lost sight of and a certain amount of research on the biology of fishes was continued. Such a preservation of a link with more direct fishery research is of value if only because it necessitates that contact shall be kept with current work elsewhere. The Association has, for instance, undertaken research on the breeding and habits of the mackerel, the distribution of seals, the effects of T.N.T. on oysters, and other problems, at the request of the Ministry of Agriculture and Fisheries. But such research should not nowadays be needed as a justification for receiving a Government grant. All fundamental researches in the long run justify themselves, and the bulk of the work done at Plymouth is essentially fundamental in nature.

Thanks to the valuable work of the Fisheries Departments our factual knowledge of the important food fishes is such that the populations of fish on which our food supply depends can now be watched with a view to the regulation of the catches. The major picture of the distribution, migrations, growth and spawning habits of the common fish is now well known. But what the underlying factors may be, on which the fishes' lives and habits depend, remain to a large degree unsolved, and their solution lies more in the realms of pure science. It seems likely, therefore, that all fisheries research will become more fundamental in nature and aimed at understanding the great natural fluctuations in the fish populations and the causes of the habits of the fish themselves.

It may be noted that most of the researches that have been undertaken in recent years by the staff of the Plymouth laboratory can be built around two main underlying themes. The first is how much living matter can the sea produce, what are the variations and causes of variation in productivity, and how do the organisms obtain the materials necessary for life? The second is how do marine animals in general live, how do they fit their various individual environments, and what alterations in the conditions in their environment can they appreciate? Both require a knowledge of the physical and chemical conditions in the sea. Where the productivity of the sea is concerned the seawater is the medium which contains all the ingredients necessary for the successful growth and development of the living organisms, in so far as the general biology of the animals is being studied it is the conditions in the sea water which determine their distribution, habits and migrations.

The Plymouth laboratory is therefore equipped for studying the physics and chemistry of the sea. Researches of the staff are aimed at the development of methods for estimating the quantities of nutrients in the sea water upon which the unicellular plants depend and following the changes they undergo throughout the year and from place to place in relation to the plant crop. The approximate yearly cycle of the more abundant constituents, phosphorus, nitrogen and silicon is now known. But much remains to be done in studying their rate of turn-over, and long-term investigations have shown periodic fluctuations in the amounts available. This is now being linked with hydrological observations on the movements of water masses and their origins, for much will depend upon whether the water is drawn

from the rich deep ocean layers upwelling on the continental shelf or from the more depleted surface waters

One of the first necessities in this research is a knowledge of the amount of photosynthesis, photoelectric methods have therefore been adapted for the measurement of the penetration of light into the sea and the extinction by absorption and scattering of its component wave-lengths at varying depths.

In order to solve some of the problems thus posed it is necessary to work with pure cultures of diatoms and flagellates, and for many years attention has been given to this side of the work   Advances have thus been made in our knowledge of the utilization of combined nitrogen and phosphorus by the plants, these constituents often being present in such exceedingly low concentrations in the sea as to limit plant growth. Research is also being made on other substances, necessary only in minute quantities as trace elements, now known to play a vital part in the growth of land plants. Thus the concentration of both iron and manganese is probably suboptimal in some waters and may indeed limit plant production.

It is on the production of these unicellular plants that all the animal life in the sea depends, and ultimately those fish which form so valuable a part of man's food supply The first link in the chain from plant to fish is the minute animal life of the plankton. Not only are these eaten directly by such fish as the herring and the mackerel, but they are of direct importance to nearly all species of fish, because when first they hatch the young fish are too small to eat anything larger. Researches on distribution, abundance, growth and habits of the many species of animals in the plankton therefore form a necessary part in the general problem of productivity. The effects of grazing off the plant crops by these animals can be studied at sea by evaluation of their numbers in measured volumes of water, and in the laboratory by experiments on the rate the animals eat the plants when cultured.

Observations on the distribution of the plankton organisms are made also in connexion with the hydrological surveys. It is found that some species are restricted to certain types of water, and they can thus be used as indicators of their respective water masses. Some waters in the Plymouth area, which are thus clearly characterized biologically, are not readily distinguishable by the usual hydrological features such as salinity and temperature. Such biologically distinguishable waters may differ markedly between one another in the amount of life they carry. This must in turn be related to their chemical content.

Other links in the chain are the bottom animals upon which the growing fish feed. It is necessary first to know the distribution of these animals. The bottom deposits of the sea are not uniformly distributed, ranging as they do from the finest mud to the coarsest gravel according to the movements of the overlying water. Each kind of deposit has its characteristic fauna; and recent researches have shown that the microscopic larval stages of some animals will only undergo their normal metamorphosis if they can find the individual grade of soil they live in. The estimation of the food available in different deposits has received attention, and attempts have been made to evaluate the animal contents of standard samples of deposit.

There is another link in this productivity chain whose connexion may not at first appear obvious. Quite early in the history of the Marine Biological Association the opportunity was taken, while studying the distribution of bottom animals, to examine also the stones and rocks dredged up in order to throw light on the geology of the English Channel. A knowledge of the configuration of the sea floor is of importance for the study of water movements. The shape of the continental edge where it passes over from the shelf to the steeper continental slope may be of critical importance, for it is here that the deeper waters of the ocean rich in nutrient salts are brought towards the surface at times by upwelling and reach the photic zone. It may well be that embayments resulting from submerged valleys may cause submarine waves to increase their amplitude and thus reach higher levels.

Let us now consider the second major line of research, the biology of marine animals. Every species of animal in the sea can form a subject for enquiry, and each alone can pose nearly all the problems of biology. The sea provides a greater variety of animals than any other environment. In it are to be found representatives of all the groups of the animal kingdom, the insects alone being scarcely represented. Many groups indeed are found almost exclusively in the sea The facilities offered by the Plymouth laboratory therefore afford an inexhaustible mine for any biologist, and it becomes difficult to canalize the work into any single objective line. There is scope for systematists, for morphologists and embryologists, for students of life histories and behaviour, for geneticists and so on. In general it may be said that, once the ground has been laid open by the systematists, research has chiefly been directed towards the description of the life histories of animals important in the general economy of the sea, of their food and methods of feeding, of their breeding and rate of growth, their parasites, and of their relationships with their animate and inanimate environment. Many of the results of investigation obviously have also a bearing on the general problem of productivity. In this wide field for research the emphasis tends to vary from one direction to another according to the predilections and aptitudes of the individual workers, but, apart from their value as contributions to general biology, any one of them can be shown directly or indirectly to have its practical bearing. Knowledge of the life histories and habits of fishes in general has obvious value, even where species of no commercial interest are concerned, since they are all competitors for the common food supply. Very useful results have accrued from investigations on the herring, which at one time formed an important winter fishery at Plymouth, attracting a hundred or more steam drifters from the east coast ports. In the early 1930's the herring ceased to come in their usual numbers to the grounds near Plymouth, and it became possible by local observations to warn the industry of the reduced chances of a successful fishery and thus save the considerable expense of sending ships to the area. The causes of the disappearance of the herring are, however, of the greater fundamental interest and the answer may be found when our knowledge of water movements grows.

Apart from the more obvious necessity of research on the biology of crabs, oysters, mussels and other shellfish used for food, knowledge gained about inverte-

brate animals in general has proved its worth. The annual cost to the nation resulting from damage to underwater structures by boring organisms and by the fouling growths on ships' bottoms is immense. All attempts to reduce this wastage by improved methods require at the start a knowledge of the natural history of the organisms concerned. Other departments are now taking up antifouling problems, but they begin with a basic knowledge already supplied by fundamental researches.

It is not wise in the long run to restrict observations only to those organisms known to be of economic interest. It has been noteworthy that our common coastal seaweeds attracted little attention in the past. In relation to the general economy of the sea as a whole the narrow fringe of weeds around our coasts is of small importance; probably largely for this reason the seaweeds were neglected. But during the war, when supplies of certain raw materials were cut off, seaweeds were needed as a source of supply of alginic acid, agar and mannitol. It was then realized that we knew practically nothing of the rate of growth and breeding of our commonest weeds, and investigations were immediately begun.

I think this necessity for the accumulation of knowledge without regard for its immediate practical value should be stressed, for it has proved itself abundantly worth while. The Association has often advised Government departments with resulting savings in expense. A knowledge of the effects of temperature on the rate of growth of marine organisms was incidental in the destruction of fouling organisms and their prevention for many succeeding years in a large basin in one of our naval dockyards. And, in passing, it is worthy of mention that research on the preservation of ropes and nets, besides enabling the Plymouth laboratory to make considerable economy in the use of expensive silk plankton nets, resulted in great saving for the Ministry of Home Security when proven methods were adopted for preserving sand bags.

But marine animals live not only in the open sea. They inhabit the intertidal zones of the shore and they invade the estuaries. Work cannot therefore be limited to offshore waters; the shores and estuaries must also receive attention. The examination of the estuarine fauna is of great interest physiologically, and a detailed knowledge of the distribution of the different species in relation to the normal changing conditions is of value in assessing pollution. As a result of a close study of a water shrimp (*Gammarus*), primarily as a subject for genetics, certain species are proving to be valuable estuarine indicators.

From the point of view of life in the sea as a whole it should be realized that work at Plymouth touches only the borders of the great oceans, whose study lies within the province of the highly organized oceanographical expeditions. Nevertheless Plymouth plays its part in the promotion of oceanographical research. This is especially so in the development of methods. Many of the methods used on ocean-going expeditions have been developed at Plymouth. This is an essential part of the laboratory's work. Once an oceanographical expedition is equipped and its programme planned it is necessary that the majority of the observations should be carried on by routine methods, for results lose comparative value if they are

constantly varied en route. At Plymouth, however, there is full opportunity to develop methods in the laboratory and test them out at sea. Each succeeding cruise by the ocean-going research vessels may therefore take advantage, and employ the improved methods and attack new problems for which the necessary technique had been awaiting development at a shore laboratory.

This brief review of the problems open to investigation shows some of the field of research available to the Plymouth staff. The scientific staff is small, only a dozen or so in number. The most that can be done is to distribute this staff in a balanced manner so that there is one engaged in each of the possible major lines of inquiry. Some might argue that it would be better to concentrate all the energy on to one specific problem. This could only be done by the formation of a school and the interests of the leaders of this school might determine a one-tracked course for many years.

This should never be at Plymouth, because there is another most important side of the laboratory's work upon which I have not yet touched. It has been a tradition of the Plymouth laboratory that it shall .attract visiting research workers. The constant flow of visitors gives life to the buildings and, with its ever changing interests, affords invaluable points of contact for the staff. It is essential, therefore, that the interests and experience of the staff should cover as wide a field as possible so that visitors may receive assistance and mutual benefit be derived.

The additions to scientific knowledge produced by the many visitors must exceed those of the staff itself and they are for the most part published in journals other than that of the Association. Much of this work has been on traditional biological lines, but it may be noted that, even on the day of the Association's foundation, the words of Romanes pointed to other fields. When the time was ripe the scope was broadened to include the comparative physiology of marine animals. In this direction Plymouth has always been understaffed, but it has for long been the aim as far as space will allow to equip the building with the necessary facilities and apparatus so that visiting workers may fill this gap.

The physiological researches made at Plymouth have been very varied, and mention only can be made here of some of the subjects which have received attention. The common spider crab (*Maia*) has been much used for studying the heat formation in nerves and other problems of the physiology of nerves; the same animal also supplies material for the study of the respiratory blood pigment, haemocyanin. The sea urchin (*Echinus*) has been a fruitful animal for experiments on fertilization and development since its eggs are most suitable in nature. Advances have been made in our knowledge of the nervous coordination of the movements of fishes and on the physiology of the regulation of their colour changes. The functions of the lateral line system in fishes have been partly elucidated, and our knowledge of the labyrinth has been advanced owing to the unique suitability of the dogfish (*Scyllium*) as a subject for experiments.

Nervous systems in their simplest form have been studied in the sea-anemone, and in recent years the squid (*Loligo*) and cuttlefish (*Sepia*) have received

prominent attention because they possess giant nerve fibres on which much can be done which is not possible with other nerve fibres; these giant fibres are also to be found in other marine animals, notably some polychaete worms and Crustacea.

Much remains to be done on the physiology of marine animals, and one direction perhaps in which our knowledge is especially lacking is that of the sensory perceptions and environment of animals in the sea.

From these few examples it can be seen that the practical benefits resulting from the founding of the Marine Biological Association may have a wider application than is expressed in its original aims, for these physiological researches have a very definite connexion with the medical sciences.

In this account I have omitted to mention by name the many distinguished men of science whose researches have resulted in the success of the Plymouth laboratory, and who, together with the Association's many devoted benefactors, have raised the laboratory to its present position of world repute. I cannot, however, let the occasion pass without reference to the late Edgar Johnson Allen who, as Director for 42 years, was the guiding genius to whom the Marine Biological Association owes so much of its success (Bidder 1943; Kemp & Hill 1943). It is interesting to recall Dr Allen's published works. These were comparatively few, but it is noteworthy that they touched on nearly all the main fields of research covered by the laboratory. To him was due the pioneer work on the culture of diatoms which made possible the great advances in our knowledge of the productivity of the sea. He produced the first important publication on the bottom fauna with his work on the Eddystone-Start grounds, and he was an acknowledged authority on the systematics of polychaete worms. His writings cover many problems concerning fish and other products of the sea. He co-operated in work on development and heredity, and had a deep interest in evolution; and it is perhaps significant that his first researches were on the nervous system of Crustacea. I think this is sufficient to show why the Plymouth laboratory never developed into a one-sided institution.

It would be unfitting also if I failed to include the name of the late Director, Stanley Kemp, whose death came as so tragic a blow just as the war was ending (Hardy 1946). Dr Kemp's name will go down in history as that of the leader of one of the greatest oceanographical expeditions of all time. The *Discovery* Expedition has become a living institution, and the loss of Dr Kemp is deeply felt by all biologists and most by the staff of the Plymouth laboratory.

I should like also to record one name out of those of the Association's many benefactors, that of George Parker Bidder, for whose wisdom, generosity, and unfailing help in times of need we shall always remain in debt.

The laboratory is managed by a Council of elected members and annual Governors appointed by certain governing universities and other bodies, including the Royal Society, which have given sums of £500 or more. A number of Universities also contribute to the Association by renting tables to which they can nominate research workers. All foreign visitors are welcomed as guests free of charge and every year

sees an increasing number of foreigners coming to Plymouth to work and discuss common interests with members of the staff.

The private sources of income of the Association are from these grants and donations, from membership subscriptions and the proceeds of the sales of specimens, collecting gear and journals. By far the largest contributor at the present time is the Government which voted an annual maintenance grant last year of over £25,000. The grant is sanctioned by H.M. Treasury as a draft from the Development Fund, and the Association is deeply in debt to the Development Commissioners, their advisers, and their Secretary, E. H. E. Havelock, for the wisdom and foresight they have always shown in their recommendations for the laboratory's support.

The Laboratory is built of Devonian coral limestone of which the Plymouth Hoe is formed, and marine animals of a past age are clearly visible in the weathered stone. It is not very large; accommodation is restricted by the limited area available between the Citadel walls and the road. It consists of a main south building with the two upper floors divided into working rooms in which about twenty-four workers can be accommodated. Connecting with it is a north block containing the chemical and physiological laboratories. Underneath are cellars excavated from the solid rock which, on account of their uniform temperature and freedom from vibration, are most suitable for research requiring very delicately adjusted apparatus. There is a small constant temperature building with two compartments for controlled low temperatures.

The north block can take about sixteen research workers. The whole laboratory can thus accommodate some forty people* at one time, although this number can always be increased by a little 'crowding up'. The present permanent scientific staff numbers twelve, and there are also usually half a dozen or so investigators on long-term grants of a year or more duration. Over and above these about twenty visiting research workers can therefore be accommodated at one time, and it is of course normal for the laboratory to be especially crowded during the summer months when University staffs are on long vacation and visitors come to this country from abroad.

There is a valuable library, which is probably the most complete in the country in publications concerned especially with marine biology and oceanography. The library also takes a number of other periodicals likely to be needed by visiting research workers. Many visitors remark on the pleasure of using a library of so compact a nature and with such careful selection of publications.

On the ground floor of the main south building there is an aquarium which is open to the public. Apart from its educational value for the many adults and children who visit the aquarium, the tanks with their living exhibits are a never failing source of interest to the research worker on the habits and behaviour of

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* The eastern block of the south building, which was the Director's house, was gutted by fire in a bomb attack. It is being rebuilt as a laboratory and should increase the working accommodation by ten rooms.
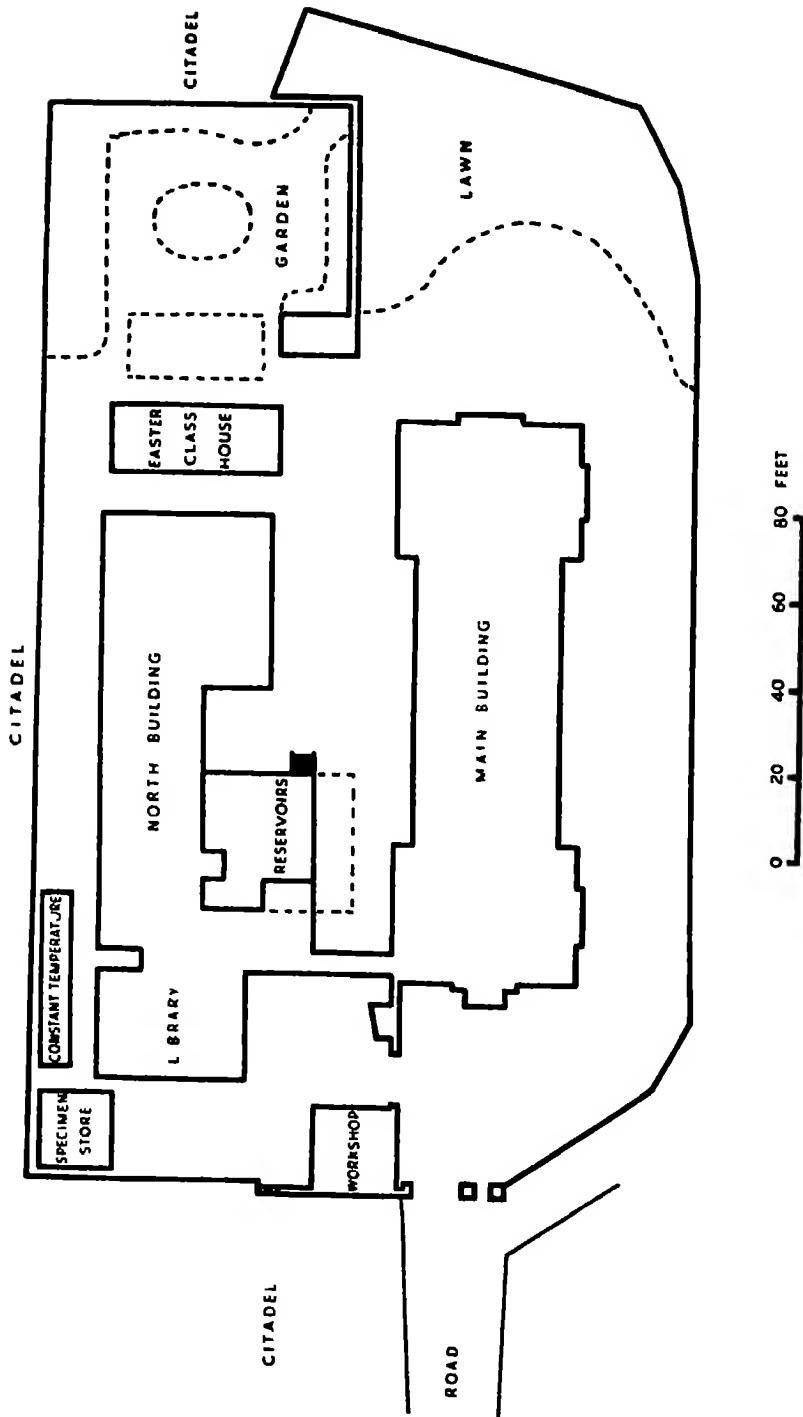
FIGURE 1 Site plan of the laboratory buildings.

animals. The exhibits are restricted to the local fauna and provide a representative view of the chief fishes and larger invertebrates of the district.

- The tanks, the largest of which is 30 ft. 6 in. long by 9 ft. wide, with a depth of water of 4 ft. 6 in., are supplied from two reservoirs each holding 50,000 gal. of sea water. This sea water which supplies the aquarium is also led to certain parts of the laboratory where small experimental tanks and seawater circulation benches are available.

A subsidiary, but nevertheless vital, function of the laboratory is the part it plays in the general training of biologists Facilities are provided for courses of instruction during the Easter vacation to university students and schools, who have unrivalled opportunities for seeing at first hand the variety of living organisms which abounds in the sea, and for studying the different environments in which they live. There can be few universities in this country whose zoology staffs do not contain a sprinkling of those who have passed some of their time at Plymouth, either as Easter Course students, members of the scientific staff, or visiting research workers. This is most important in view of the overwhelming preponderance of animal types in the sea.

From time to time more specialized courses are given for post graduate students on the physiology of marine animals, and other special subjects. Plymouth also plays another part in education by supplying to universities and schools preserved and living specimens necessary for teaching purposes

In order to enable the demands of these manifold activities to be met the Association runs two research vessels. The smaller of these, a 25 ft motor boat, the *Gammarus*, is used for dredging and trawling in waters close inshore, and for visiting the shores at different points for collecting intertidal specimens. The larger, at present a 90 ft. motor fishing vessel (R.V. *Sabella*) on charter from the Admiralty, works in offshore waters. As well as making collections generally for those working in the laboratory, for stocking the aquarium, and for the specimen trade, the first call on this vessel is naturally for research at sea It is from this ship that quantitative observations are made on the organisms of the plankton and the sea bottom, and from which physical and chemical investigations are made at sea. The ship also makes periodical cruises further afield to study the hydrology over a larger area including the western approaches to the English Channel

I hope I have said enough to give some idea of the general activities of the Plymouth laboratory and the possibilities it affords for work. One might sum it up by saying that it aims to give facilities for any research, not necessarily only biological, on problems for which the sea can provide the materials or the environment required. Its position is unique, lying as it does between the extremes of a fishery research laboratory and of an oceanographical institution, yet serving both, and at the same time offering facilities for visitors like the laboratory at Naples on whose pattern it was first founded. Let us hope it may be allowed to continue to hold this focal position and attract all those interested in the science of the sea and indeed of life itself.

Note. There have been extensive alterations to the Plymouth laboratory since the account written by Allen & Harvey (1928). A new library was built in 1931, and in 1932 the north block was further extended to the eastward, to give increased accommodation for physiological and chemical laboratories, and improved photographic darkroom facilities. A small constant temperature building was added in 1938. In 1939 the centre of the south building, the original main laboratory, was completely renovated and a new floor added. A site plan as at the present date is shown in figure 1.

I wish to thank Miss E. J. Batham, Mr D. P. Wilson and Mr G. A. Steven for permission to reproduce the photographs.

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DESCRIPTION OF PLATES 2 TO 8

PLATE 2

FIGURE 2. View of the Plymouth marine biological laboratory, taken from the Smeaton Tower on the Hoe looking eastwards towards the Cattewater (summer, 1946).

PLATE 3

FIGURE 3. Tank room on the first floor of south building (south side).

FIGURE 4. Tank room on first floor of south building (north side).

Figure 2

Figure 3
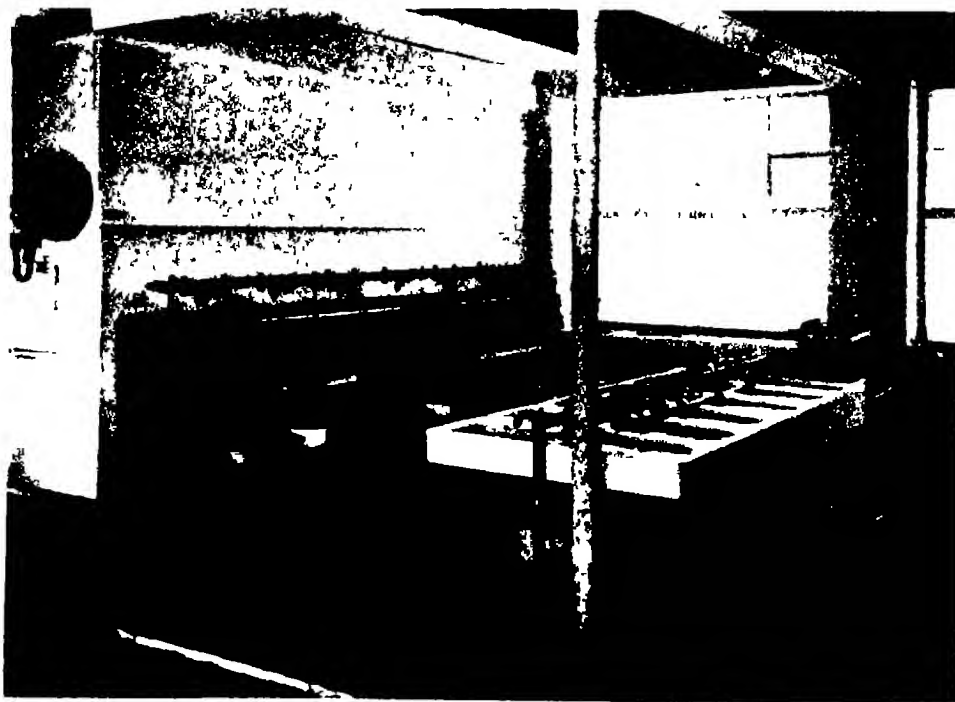
Figure 4

Russell

Proc. Roy Soc. B, volume 135, plate 4

FIGURE 5

FIGURE 6

Russell

Proc. Roy. Soc. B, volume 135, plate 5

FIGURE 8

FIGURE 7

Russell

Proc. Roy. Soc. B, volume 135, plate 6

FIGURE 9

FIGURE 10

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Proc. Roy. Soc B, volume **135**, *plate* 7

Figure 11

Figure 12

FIGURE 13

FIGURE 14

PLATE 4

FIGURE 5. Museum on second floor of south building.
FIGURE 6. Typical work room in south building.

PLATE 5

FIGURE 7. Reading room on first floor of library.
FIGURE 8. Corner of physiological laboratory.

[PLATE 6

FIGURE 9. Chemical laboratory.
FIGURE 10. Yard between north and south buildings showing reservoirs for sea water on left, outside tanks and circulation bench on right, and end of Easter Course building in distance on left.

PLATE 7

FIGURE 11. General view of aquarium.
FIGURE 12. Anemone tank in aquarium.

PLATE 8

FIGURE 13. R.V. *Sabella*.
FIGURE 14. Motor Boat *Gammarus*.

The photograph for figure 2 was taken by Miss E. J. Batham. All the other photographs are the work of D. P. Wilson, except figures 13 and 14 which were taken by G. A. Steven.

CROONIAN LECTURE

The antidiuretic hormone and the factors which determine its release

BY E. B. VERNEY, F.R.S., *Department of Pharmacology, University of Cambridge*

[Plates 9 to 11]

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When water is given under specified conditions to the living dog, there is a lag of 15 min between the peak of the animal's water load and the maximum rate of water excretion by the kidney. The diuresis is inhibited by emotional stress, the inhibition being of post-pituitary origin and itself suppressed by an immediately preceding injection of adrenaline or tyramine. Reasons are given for the view that this action of adrenaline is independent of any accompanying increase in arterial pressure or cerebral blood flow. The intracarotid injection (short period, 5 to 20 sec.) of 'hypertonic' solutions of sodium chloride (but not of urea) causes a similar inhibition, the response being diminished by some 90 % after removal of the posterior lobe. The response to sodium chloride is shown to be osmotically determined, and the term 'osmoreceptors' has therefore been introduced as descriptive of the autonomic receptive elements with which the neurohypophysis is functionally linked. The receptors are somewhere in the vascular bed of the internal carotid artery. The results of long-period (10 and 40 min.) intracarotid infusions and of the short-period injections, show that the osmoreceptors are freely permeable to urea, less freely permeable to dextrose and relatively impermeable to sodium chloride and sucrose. The local increase in osmotic pressure required in the 40 min. infusions (unilateral) to reduce the urine flow during water diuresis to about 10 % of its maximum, is some 2 % only; and assay of such response shows it to have a post-pituitary extract equivalence of $1 \mu U$/sec. $(0.5 \times 10^{-9}$ g /sec. in terms of the standard powder). These facts give an intelligible interpretation to the time lag between the peak of the water load and the peak of diuresis. Water diuresis, therefore, is fitly and accurately described as a condition of physiological diabetes insipidus, the antidiuretic secretion of the neurohypophysis being a hormone in the sense that its liberation is continually governed by the concentration of sodium chloride, and of other osmotically active substances, in the arterial plasma.

I INTRODUCTION

The experiments which it is my privilege and main purpose to describe in this lecture are all based on the phenomenon of water diuresis in the dog, i.e. the profuse diuresis which follows the oral administration of an adequate volume of water to the living animal; and in order that they may be viewed on their proper background of fact and idea, it is fitting to give first an outline of earlier work, not only on the phenomenon of water diuresis itself, but also on other phenomena which are intelligibly related thereto.

The discovery some forty years ago (Schäfer & Magnus 1901; Schäfer & Herring 1906) that extracts of the infundibular portion of the hypophysis had both diuretic and antidiuretic properties led to Schäfer's (1909) subjecting the pituitaries of three dogs to injury or partial destruction by means of a feeble thermocautery: in all, a polyuria supervened for some period during the 4 to 11 post-operative days over which the animals were being observed. Doubtless the disclosure, by his experiments on the anaesthetized dog, of the sway held by the diuretic over the antidiuretic activity of post-pituitary extract, prompted the caption 'Stimulation of pituitary by injury' to the description of the results of his survival work on the dog; but the fact remains that these results gave the first clear indication of functional linkage between the kidney and the pituitary. A few years later Frank (1912) published his clinical observation of the frequent association of diabetes insipidus with injuries to the hypophysis; and, following Schäfer's interpretation of the results of his experiments on the dog, Frank expressed the view that diabetes insipidus in man was attributable to pathological overactivity of the pars intermedia. The following year, however, v. den Velden (1913) and Farini (1913) demonstrated the efficacy of injections of post-pituitary extract in relieving the signs and symptoms of the human disease, and v. den Velden was clearly puzzled by the conflict between his results and the current interpretation of Schäfer's experimental findings. Evidence has since been gradually accumulating that the polyuria frequently associated with experimental or pathological lesions of the pituitary and hypothalamus is to be ascribed rather to lack of post-pituitary anti-diuretic substance than to increased secretion of Schäfer and Magnus's diuretic substance, evidence which has culminated in the elegant demonstration by Ranson and his colleagues (Ranson, Fisher & Ingram 1938) that, in the cat and the monkey, the occurrence of diabetes insipidus is contingent upon the complete or quasi-complete degeneration or removal of the neurohypophysis. Using the Horsley-Clarke stereotaxic instrument, they have been able to place small discrete lesions at any desired points in the hypothalamus, and to show that when by these means the supraoptico-hypophysial tracts are interrupted, a series of striking degenerative changes takes place in the supraoptic nuclei and in the neural division of the hypophysis, the degeneration being associated with a fall of some 95 % in anti-diuretic activity as measured by comparative assays of extracts of the normal and of the atrophic pituitaries. Under these conditions the pars intermedia retains histological normality; and this fact, coupled with evidence derived from measure-ment of the antidiuretic activity of extracts of (1) the separated pars nervosa and pars intermedia (Van Dyke 1926), (2) the bird's hypophysis in which the pars intermedia is absent or ill defined (Geiling & Robins 1938, De Lawder, Tarr & Geiling 1934), and (3) the pars nervosa of the whale in which the pars nervosa is entirely separate from the pars distalis or anterior lobe (Geiling & Robins 1938), shows that the chief if not the only source of post-pituitary antidiuretic substance is the neural lobe and neural stalk, i.e. the neurohypophysis.

A second condition under which a profuse watery diuresis is observed is perfusion

of the dog's kidney in the isolated state (Verney & Starling 1922; Starling & Verney 1925). Here the diuresis is inhibited by the addition of post-pituitary extract to the perfusing blood, the inhibition being specific in the sense that it is accompanied by an increase both in the concentration of chloride in the urine and in the rate of chloride excretion, and that it may occur without appreciable diminution of blood flow through the kidney at constant perfusion pressure. Moreover, these effects of post-pituitary extract are closely simulated by switching into the perfusion circuit, in parallel with the kidney, the head of a dog, the inhibition of diuresis and increased excretion of chloride being then dependent upon the presence of the pituitary in the perfused head (Verney 1926). There can, I think, be little doubt that the view put forward at the time, viz. that the phenomenon of profuse watery diuresis exhibited by the kidney perfused in the isolated state is due to the divorce of the kidney from the inhibitory influence of post-pituitary antidiuretic substance, is substantially correct.

The view, therefore, that diabetes insipidus derives essentially from a deficiency, complete or extreme, in the antidiuretic product of the neurohypophysis would appear, on the available evidence, to be incontrovertible.

There is yet a third condition under which a profuse watery diuresis appears, viz. the administration of water by mouth to the living mammal; and the question arises as to whether this phenomenon, too, is of pituitary origin. In this connexion it has been established (Heller & Smirk 1932; Klisiecki, Pickford, Rothschild & Verney 1933a) that alimentary absorption is well in advance of diuresis. Klisiecki et al. obtained a measure of this in the dog, and showed that under the conditions of their experiments there was an interval of 15 min. between the peak of the water load and the peak of diuresis: the significance of this interval will be considered later. Further, the diuresis response of the denervated kidney runs strictly parallel with that of the innervated kidney (Bykow & Alexejew-Berkmann 1931, Klisiecki et al. 1933a), and the response is unaccompanied by any change in the volume flow of blood through the kidney (Cowan, Verney & Vogt 1938). Next, this diuresis response may be inhibited in various ways: by intravenous injection of adrenaline or of post-pituitary extract, by the withdrawal of small volumes of arterial blood, and by subjecting the animal to short-lived muscular exercise or to emotional stress. The inhibitory effect of adrenaline is characterized by the abruptness of its onset, by its brevity and its rapidity of disappearance (Theobald & Verney 1935; Rydin & Verney 1938), and by a well-marked and fleeting fall in renal blood flow (Cowan et al. 1938), while that of post-pituitary extract is characterized by being less abrupt in onset, more prolonged in course, and more gradual in disappearance than that of adrenaline (Theobald & Verney 1935; Rydin & Verney 1938), and by an absence of change in renal blood flow (Cowan et al. 1938); moreover, the response of the innervated kidney to post-pituitary extract runs closely parallel with that of the denervated kidney (Klisiecki et al. 1933 a). The effect of the withdrawal, during water diuresis, of small volumes of arterial blood from dogs in which the abdominal splanchnic nerves had been divided, or in which this division had been combined

with removal of the abdominal sympathetic chains, was investigated by Rydin & Verney (1938): the urine flow fell fairly precipitously to a period of slow recovery reminiscent of the response to post-pituitary extract. Seeing that the induced change in arterial pressure was small and fleeting, and that, when the renal blood flow was temporarily reduced through the operation of a compression unit implanted at the origin of the renal artery, the inhibition of urine flow was in temporal accord with the period of reduced blood flow, the conclusion was reached that the inhibitory effect of haemorrhage was humorally determined by some agent other than adrenaline. The suggestion that this agent is post-pituitary antidiuretic substance has not as yet been put to the test of experiment. The analysis of the inhibitory effect of short-lived muscular exercise and of emotional stress, however, has been carried further, and while that of the former has shown it to be due to its emotional accompaniment (Rydin & Verney 1938), that of the latter has given the first demonstration of the release of post-pituitary antidiuretic substance in the living animal when subjected to an appropriate physiological stimulus. It will be apposite, therefore, to consider the inhibitory effect of emotional stress in some detail

II. The release of post-pituitary antidiuretic substance by emotional stress

In the reference by Bernard (1859) to the fall in the rate of urine secretion during the emotional stress associated with operative procedures on the human subject, the suggestion is implicit that the fall is of vasomotor origin, and Mackeith, Pembrey, Spurrel, Warner & Westlake (1923) attributed to a similar cause the decrease in urine flow produced by a 'feigned run for 3 or 4 min. during which the tense attitude for starting to run was maintained'. Klisiecki *et al* (1933 *b*), however, observed in a dog, in which the left splanchnic nerves had been divided and the ureters exteriorized, that on its exposure during water diuresis to a cat, a temporary fall in the rate of urine flow ensued, the fall being about equally marked on the two sides, and Theobald (1934) showed in the dog that afferent nerve stimulation produced, during water diuresis, an inhibition the course of which was of the pituitary type. Subsequent work has demonstrated the inhibition's essential independence of the renal nerves (Theobald & Verney 1935; Rydin & Verney 1938), and of the endogenous release of adrenaline (Rydin & Verney 1938).* Moreover, while the course of the inhibitory response to emotional stress was found to be not essentially changed by successive operations comprising (*a*) division of the renal nerves,

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* In the later experiments of Rydin and myself emotional stress was produced by the stimulus of a weak faradic current from the secondary coil of a Palmer inductorium fed by a 2 V battery in the primary circuit. The current was carried to the subcutaneous tissues of the dorsum of the lumbar region by surgically clean needle electrodes, and the strength of stimulus was increased until the animal showed signs of resentment. I have used this form of stimulus in all subsequent work in which the production of such stress was required.

(*b*) removal of the right suprarenal gland and denervation of the left, and (*c*) decentralization of the whole abdominal sympathetic system with removal of ganglia L1 to S1 inclusive, records of the arterial blood pressure (obtained from the femoral artery under local anaesthesia) during these inhibitory responses showed that after (*a*) and (*b*) the mean arterial pressure rose during the emotional stress, and that after (*c*) it remained unaffected. The inference was drawn that the inhibition of urine flow by emotional stress was independent of change in renal blood flow, and the correctness of this inference has since been demonstrated by actual measurement of the renal blood flow by an application of Rein's (1928, 1929 *a, c*, 1931) thermo-stromuhr method (Cowan *et al.* 1938). While, therefore, the ascription of the inhibition of water diuresis by emotional stress to known and possibly competent physiological events had failed, this very failure served to increase the likelihood of the inhibition being of pituitary origin. Indeed, the candidature of post-pituitary antidiuretic substance for this role had now become strong witness the minuteness of its minimal effective antidiuretic dose; the innervation of the neurohypophysis by fibres from the supraoptic and paraventricular nuclei, the release of post-pituitary antidiuretic substance by acetylcholine in the atropinized dog (Pickford 1939); and the lag in onset and external resemblance of the inhibitory responses to intravenously administered post-pituitary extract and to emotional stress, the responses becoming, with a suitable dose of extract, indistinguishable.

So close a resemblance between the two responses, both in form and in measurable accompaniments and independencies, made one confident that removal of the posterior lobe in the dog would be followed by a big diminution in the inhibitory response to emotional stress. This was found to be so (O'Connor & Verney 1942). Comparison of the post-pituitary extract equivalent of the emotional inhibition before with that after removal of the posterior lobe showed that only about 5 % of the antidiuretic function of the neurohypophysis, as expressed in the inhibition by emotional stress, remained when the posterior lobe had been removed. There can, then, be little doubt that the stimulus produces its effect by ultimate involvement of the supraoptic group of hypothalamic cytons, whose axons pass down the stalk to the pars nervosa, and through whose activity the pituitary antidiuretic substance is released.

After removal of the posterior lobe, there was an increase by some 500 % in the daily output of urine for 2 to 4 days. The increase then gradually subsided, and the output was apparently normal from about a week-after the operation. This temporary polyuria had already been observed by Fisher, Ingram & Ranson (1938) working on the cat, and by Pickford (1939) working on the dog; and in experiments on the chloralosed dog (Verney 1929) removal of the pituitary body was usually followed by a rapidly appearing polyuria. It is remarkable that as much as 95 % of the antidiuretic activity of the neurohypophysis, as assessed in the emotional inhibition of water diuresis, can be abolished without producing such an impairment in the animal's ability to conserve its tissue water as would be expressed in a persisting increase in urine flow.

A. *The prevention, by increased activity of the sympathetic system and by adrenaline, of the emotional release of post-pituitary antidiuretic substance.*

In further work on the inhibition of water diuresis by emotional stress in dogs with intact sympathetic system, O'Connor & Verney (1945) distinguished a second type of inhibition, this being characterized by its rapidity in onset and by its evanescence. This response was abolished by section of the splanchnics and denervation of the kidneys and suprarenals ('denervation'), and after this the slow prolonged inhibition of pituitary origin invariably appeared. Now it was found that this slow prolonged inhibition was prevented by the intravenous injection of adrenaline just before the application of the faradic stimulus which was used to produce the emotional stress. Such injection of adrenaline, however, failed to diminish the inhibition produced by an intravenous injection of post-pituitary extract. The conclusion was reached, therefore—on the assumption that the substance' in the extract had the same structural form as had that released endogenously—that after an injection of adrenaline the resultant absence of the slow prolonged inhibition, otherwise produced by emotional stress in the 'denervated' animal, was owing to failure of release of the antidiuretic substance, and not to failure of the released substance to act on the kidney. The irregular appearance of the slow inhibition in dogs with intact sympathetic system thus became explicable in terms of an increase in sympathetic activity during emotional stress inhibiting in varying degree the liberation of antidiuretic substance from the pars nervosa; and the question was left open as to whether or no the prevention of such release were a specific effect of adrenaline. I have since made experiments with a view to contributing towards the analysis of the phenomenon. These and all the subsequent experiments described in this lecture were performed in a room essentially similar in construction and equipment to that described by Hart & Verney (1934), but ventilated by an exhaust fan instead of the airflow across the opening of the inlet duct. The large fan by which the air in the chamber could be kept circulating was not used, neither was the room temperature thermostatically controlled, the temperature, however, always lay between 19 and 22° C.

B. *The prevention, by tyramine, of the emotional release of post-pituitary antidiuretic substance.*

It was first of interest to see whether tyramine in suitable dosage was effective, like adrenaline, in preventing the emotional release of post-pituitary antidiuretic substance. The experiments were made on 'Nicky', whose operation history is outlined on p. 56, and whose photograph is given in figure 12, plate 10. Before 'denervation' (29 March 1944) this animal, during water diuresis, responded to faradic stimulation (through needle electrodes inserted into the subcutaneous tissue of the flanks) by the rapid evanescent inhibition of urine flow exemplified in figure 1 a; after 'denervation' the animal responded by the slow prolonged inhibition

shown in figure 1 *b* (graph *A*), the latter response being found to correspond closely with that to the intravenous injection of 2 mU (i.e. 2×10^{-4} c.c.; see p. 48) of pituitary (posterior lobe) extract (figure 1 *b*, graph *B*). When tyramine HCl was injected intravenously in a dose of 2·0 or 2·5 mg., it produced a transient inhibition of urine flow (see figure 3 *c*) but did not affect appreciably the response to post-pituitary extract injected 30 sec. after the injection of the tyramine (figure 2). In these respects, therefore, the effects of tyramine are similar to those of adrenaline. The fact that adrenaline (20 μg. of the base in the form of adrenaline tartrate), injected intravenously 30 sec. before a faradic stimulus, prevented the appearance of the prolonged inhibition of pituitary origin which resulted from faradic stimulation alone, was then confirmed in this animal. Tyramine HCl (2·5 mg.) was found to act in a similar manner, but this result was made equivocal by the fact that when, on the following day, adrenaline (20 μg.) was injected 15 sec. *after* the faradic stimulus, no prolonged inhibition of urine flow appeared. It seemed probable,
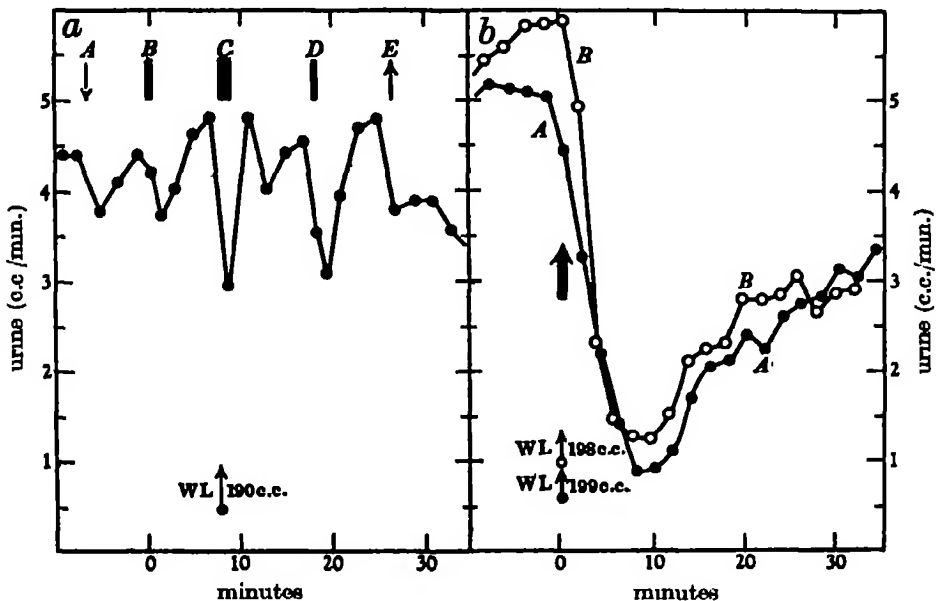
FIGURE 1. 'Nicky', in Pavlov stand. *a*, 15 March 1944. *A*, electrodes inserted, one on each side of the lumbar vertebral spines, into the subcutaneous tissue just anterior to the iliac crests. *B*, three 5 sec. faradic stimuli, coil 8·2 cm. *C*, 60 sec. stimulus, coil 8·2 cm. *D*, three 3 sec. stimuli, coil 7·5 cm. *E*, electrodes withdrawn. *WL*, water load, i.e. the difference between the volume of the test dose of water and that of the urine secreted since the giving of the test dose. *b*, graph *A* (6 April 1944) shows the response to 30 sec. faradic stimulus, and graph *B* (2 May 1944) the response to the intravenous injection of 2·0 mU pituitary (posterior lobe) extract over a period of 35 sec., the stimulus and injection being given at the arrow. In this figure and in figures 2, 3, 4 and 6 the graphs represent the urine-flow responses to the test dose of water (350 c.c.) given by stomach tube 45 to 60 min. before zero time, a hydrating dose of 250 c.c. having been given 105 to 123 min. before the test dose. In these and all subsequent experiments the urine was collected continuously, by catheter, into a series of graduated glass tubes. The rate of urine flow is plotted in the middle of the period of the sample's collection.

therefore, that an effective regeneration of sympathetic nerves had by this time,
(22 April 1944) occurred; and this was confirmed by the findings that a faradic
stimulus alone now had no appreciable influence on the urine flow, and that this
stimulus produced a rise in arterial pressure from 148 to 180 mm. Hg. The arterial
pressure was measured by means of a pneumatic cuff applied to the left carotid loop
(Verney & Vogt 1938), and the further observation was made that occlusion of the
right carotid caused the pressure to rise from 148 to 190 mm. Hg. It was decided,
therefore, to postpone further work on this animal until after complete bilateral
excision of the thoracic sympathetic chains and splanchnic nerve roots. This was
completed on 26 May 1944, and observations were resumed 10 days later.
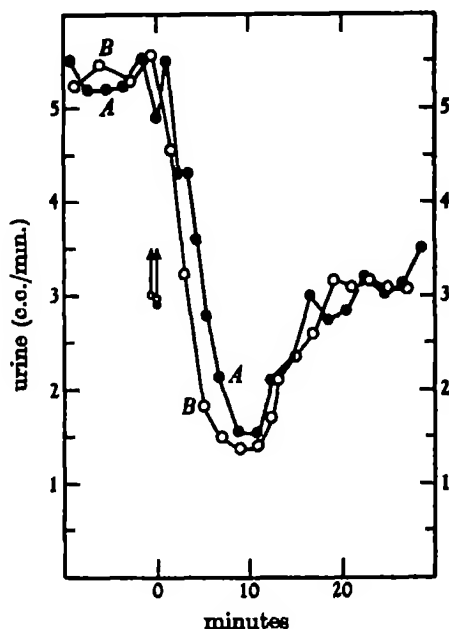
Figure 2. 'Nicky', in Pavlov stand. Graph *A*, 28 April 1944; at the second arrow 1·0 mU
post-pituitary extract intravenously. Graph *B*, 18 April 1944; at the first arrow 2·0 mg.
tyramine HCl, at the second 1·0 mU post-pituitary extract intravenously, the injections being
separated by 30 sec.

In figure 3 are shown responses to faradic stimulation 30 sec. after an intravenous
injection of 1 c.c. 0·85 % NaCl (figure 3*a*), to faradic stimulation 30 and 40 sec.
after an intravenous injection of 2·5 mg. tyramine HCl (figure 3*b*), to tyramine*

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* The course of the vascular response to tyramine was readily followed in this experiment
by observation of the prolapsed nictitating membranes. The membrane began to pale 20 sec.
after the injection, had become very pale 20 sec. later, and remained in this state for a period
of 4 mm. The pallor then gradually diminished, and the original degree of vascularity was
regained some 7·5 min. after the time of injection. The figure shows that the urine-flow
response follows a parallel time course. In another experiment the vascularity of the membrane
was watched during two large inhibitions of urine flow, each produced by the intravenous
injection of 3 mU post-pituitary extract. In neither instance was any change detected.

HCl alone (figure 3c), and to faradic stimulation 30 sec. after an intravenous injection of 20 μg. adrenaline (figure 3d). Tyramine HCl in a dose of 2·5 mg. is seen to be at least as effective as 20 μg. adrenaline in preventing the inhibitory action on

FIGURE 3. 'Nicky', in Pavlov stand. *a*, 6 June 1944; at *X*, 1·0 c.c. 0·85 % NaCl intravenously; at *Y*, two 1 sec. faradic stimuli followed by 30 sec. stimulus of weaker intensity. There was an interval of 30 sec. between the end of *X* and the beginning of *Y*. *b*, at *X*, 2·5 mg. tyramine HCl intravenously; at *Y* two 1 sec. faradic stimuli followed by 30 sec. stimulus of weaker intensity. There was an interval of 40 sec. (graph *A*, 10 June 1944) and of 30 sec. (graph *B*, 6 June 1944) between the end of *X* and the beginning of *Y*. *c*, 10 June 1944; at *X*, 2·5 mg. tyramine HCl intravenously. *d*, 9 June 1944; at *X*, 20 μg. adrenaline intravenously; at *Y* two 1 sec. faradic stimuli followed by 30 sec. stimulus of weaker intensity. There was an interval of 30 sec. between the end of *X* and the beginning of *Y*.

the urine flow of an immediately succeeding faradic stimulus. Seeing that such a dose of tyramine does not appreciably alter the response to an immediately succeeding injection of post-pituitary extract (figure 2) the conclusion is justified that tyramine prevents, as does adrenaline, the release of antidiuretic substance by faradic stimulation alone. The effect of adrenaline in this respect is, therefore, not a highly specific one.

In this animal it was now possible to show, as was to be expected, that an injection of adrenaline immediately *following* a faradic stimulus in no wise interfered with the inhibitory effect of the stimulus alone. This is demonstrated in figure 4, where the results of three experiments are depicted. In the first (figure 4*a*) are shown the effects of a faradic stimulus followed, after an interval of 40 sec., by an injection of 1 c.c. 0·85 % NaCl; in the second and third (figure 4*b*) those of a similar stimulus followed, after an interval of 47 sec. (graph *A*) and of 23 sec. (graph *B*), by an injection of 20 μg. adrenaline.
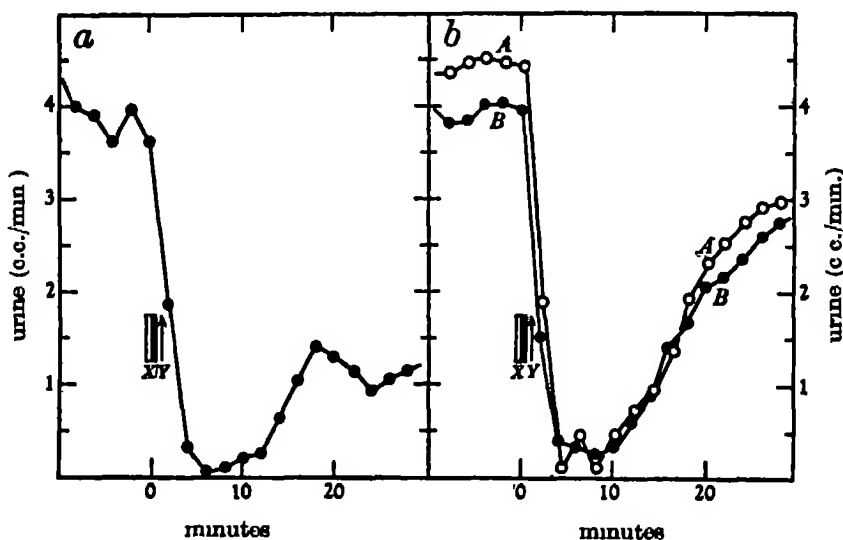
FIGURE 4. 'Nicky', in Pavlov stand. *a*, 8 June 1944; at *X*, two 1 sec. faradic stimuli followed by 30 sec. stimulus of weaker intensity; at *Y*, 1·0 c.c. 0·85 % NaCl intravenously. There was an interval of 40 sec. between the end of *X* and the beginning of *Y*. *b*, at *X*, two 1 sec. faradic stimuli followed by 30 sec. stimulus of weaker intensity; at *Y*, 20 μg. adrenaline intravenously. There was an interval of 47 sec. (graph *A*, 8 June 1944) and of 23 sec. (graph *B*, 9 June 1944) between the end of *X* and the beginning of *Y*.

The question now arises as to whether the inhibitions, by adrenaline and tyramine, of the release of pituitary antidiuretic substance have, as their common mediating cause, a rise in arterial pressure, or derive from some other and more immediate activity dependent upon the molecular configuration of these two substances.

C. *Comparison of the competency of adrenaline with that of a rise in arterial pressure from occlusion of the carotid arteries, in preventing the emotional release of post-pituitary antidiuretic substance*

For this investigation 'Nicky' was again used, and the maintained absence of sympathetic activity in this animal was demonstrated repeatedly during the period over which the observations were carried: an inhibition of the pituitary type was obtained whenever an appropriate stimulus was applied. Moreover, on four occasions the arterial pressure was being followed, before and during the stimulus, by means of a cuff applied to the left carotid loop: no rise in pressure occurred; indeed, on three occasions a small fall (6, 4 and 10 mm. Hg) was encountered. Neither carotid sinus in this animal had been denervated; and although the left carotid blood flow was inevitably reduced during the blood-pressure measurements, the rise in pressure associated with the technique of measurement would necessarily be small. This was expressed in the fact that the pressure so measured was always about 125 mm. Hg, whether or no the left carotid were occluded above the cuff. Now it was found that occlusion of both carotids produced a rise in pressure of 33 mm Hg (mean of seventeen observations, the extreme values being 22 and 50 mm. Hg), and 5µg. adrenaline, a peak rise of 28 mm. (mean of eight observations, the extreme values being 25 and 36 mm. Hg). These results were not appreciably affected by a change in the animal's posture from standing to lying on its side. It was thought advisable to test the accuracy of the cuff measurements by taking a record of the femoral arterial pressure under local anaesthesia and with asepsis precautions. The technique was as described by Verney & Vogt (1938), with the exception that heparin (1 mg./c.c. 0·9 % NaCl) was used to prevent coagulation in the fine metal cannula. The results are shown in figure 5. The increases in pressure from occlusion of the left carotid, of the right carotid and of both were 7, 10 and 38 mm. Hg respectively (figure 5, *1, 2, 3*), and the maximum increases from two intravenous injections of 5 µg. adrenaline were 29 and 28 mm Hg (figure 5, *4* and *7*). A faradic stimulus a little greater than the maximum of those otherwise employed was unaccompanied by any change in pressure during the period of the stimulus (figure 5, *6*). The results confirm the cuff measurements, and the conclusion is justified that the rise in arterial pressure from 5 µg. adrenaline is not greater than that from occlusion of both carotid arteries. Moreover, when a faradic stimulus was applied to the animal while both carotid arteries were occluded no change in the raised pressure was detected during the period of the stimulus.

In this animal occlusion of the left carotid gave an increase in the frequency of the pulse of some 5/min., of the right carotid of some 17, and of both carotids an increase of some 40/min. In another animal ('Jock', see p. 56) in which thoracic and abdominal sympathectomy had been carried out, but in which during the formation of the left carotid loop the left sinus had been denervated, occlusion of the left carotid gave no change in the frequency of the pulse, and of the right carotid an increase of 13/min., and this increase was unaffected by occlusion of the

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left carotid as well. Similarly, while no change in blood pressure resulted from occlusion of the left carotid, there was an increase of 20 mm. Hg from occlusion of the right, and this increase was unaffected by occlusion of the left as well. In the sympathectomized animal, therefore, the pressure effect of carotid occlusion is apparently closely related with the cardio-acceleration effect; and in the latter effect the potentiation of the action of one sinus nerve by that of the other points to an overlap, in the vagal (cardio-inhibitory) motoneurone pools, of the subliminal fields of excitation deriving from transmitted activity in the two sinus nerves.
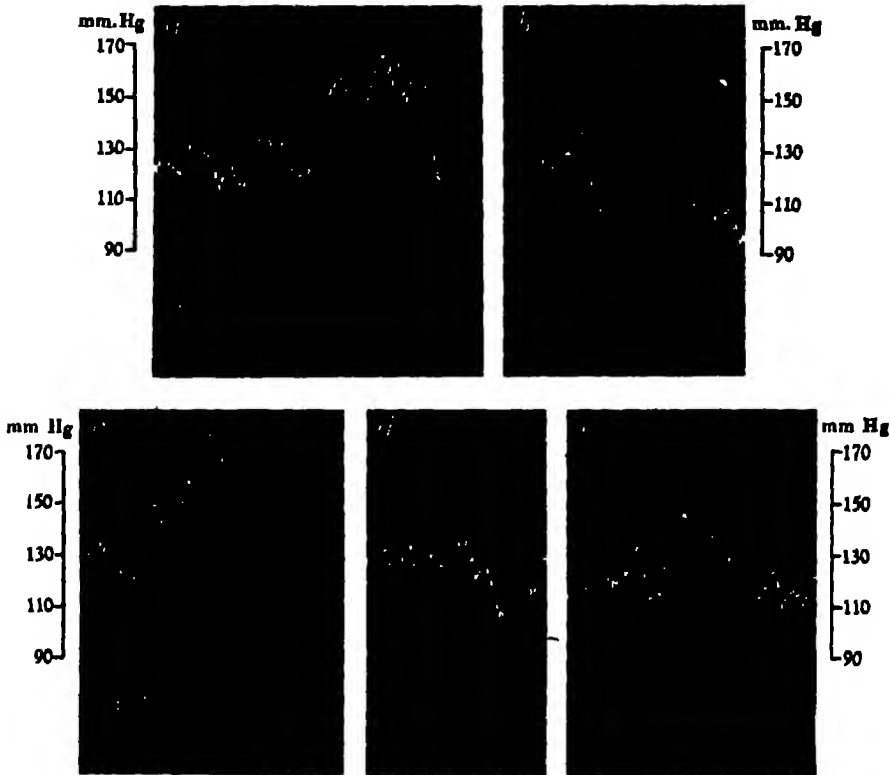
FIGURE 5. 'Nicky'. 5 November 1946. Blood-pressure record from saphenous branch of left femoral artery under local anaesthesia. *1*, occlusion of left common carotid; *2*, occlusion of right common carotid; *3*, occlusion of both carotids; *4* and *7*, 5 µg. adrenaline intravenously; *5*, 10µg. adrenaline intravenously; *6*, faradic stimulus. Between the sections *a, b, c, d* and *e* were intervals of 4, 2, 5 and ½ min. respectively.

Seeing that, in 'Nicky', adrenaline could not be a causal component in the pressor action of occlusion of the carotids, and that the rise in blood pressure from occlusion of the carotids was at least as great as that from the intravenous injection of 5 µg. adrenaline, it was of interest to compare the competency of 5 µg. adrenaline with that of carotid occlusion in preventing the emotional release of post-pituitary anti-diuretic substance. The results of this comparison are illustrated in figures 6 and 7.
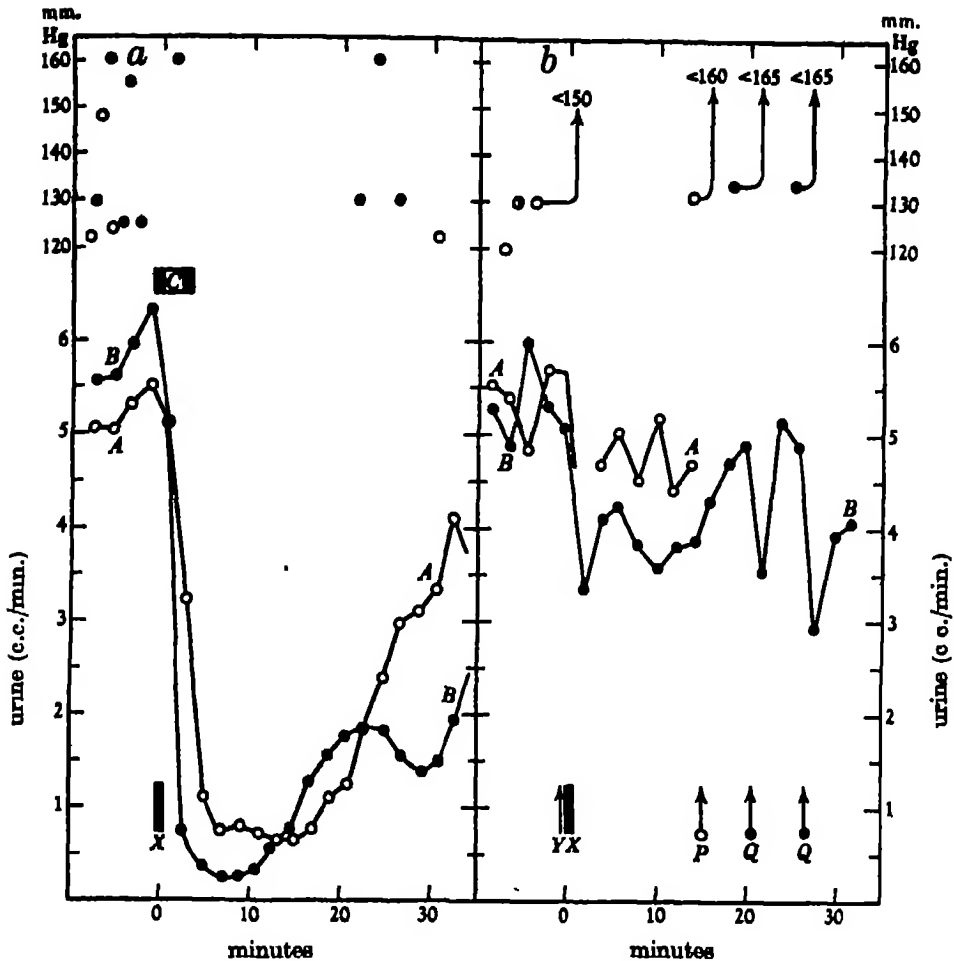
FIGURE 6. 'Nicky', in Pavlov stand. *a*, at *X* three 1 sec. faradic stimuli followed by 30 sec. stimulus of weaker intensity. Graph *A*, 10 July 1944. Graph *B*, 16 May 1945. During the period *C* of graph *B* both common carotid arteries were occluded. The blood-pressure measurements, by cuff on the left carotid loop, are charted at the top of the figure, the upper series being obtained during occlusion of the right carotid artery. During the measurements on 10 July (open circles) the left carotid was not occluded above the cuff, during those on 16 May (black circles) it was occluded. *b*, at *Y*, 5 μg. adrenaline intravenously, and at *X* three 1 sec. faradic stimuli followed by 30 sec. stimulus of weaker intensity. Graph *A*, 18 May 1945. Graph *B*, 21 May 1945. Blood-pressure measurements, by cuff on the left carotid loop, the carotid being occluded above the cuff, are charted at the top of the figure. At *P*, 5 μg. adrenaline intravenously during experiment *A*, and at *Q* 5 μg. during experiment *B*. In experiment *A* the blood pressure following *Y* and during *X* did not rise above 150 mm. Hg, and following *P* it did not reach 160 mm. Hg. Following *Q* in experiment *B* the blood pressure did not reach 165 mm. Hg. The water loads at zero time in all four experiments were between 196 and 207 c.c. The experiments show that while the rise in blood pressure accompanying carotid occlusion fails to suppress the inhibition of water diuresis by emotional stress, adrenaline in a dose which produces a smaller rise either suppresses or markedly diminishes it.

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Tests of the effect of carotid occlusion alone for periods up to 10 min. showed that such occlusion produced no fall in the rate of urine flow during water diuresis. In figure 6a, graph A, is shown the inhibitory effect in water diuresis of a faradic stimulus applied during the period X, and in graph B the effect of a similar stimulus (X) applied while the carotids were occluded (C). The blood-pressure readings during the experiments A and B of figure 6a are given at the top of the figure, the lower series being the readings when the right carotid was free, the upper when
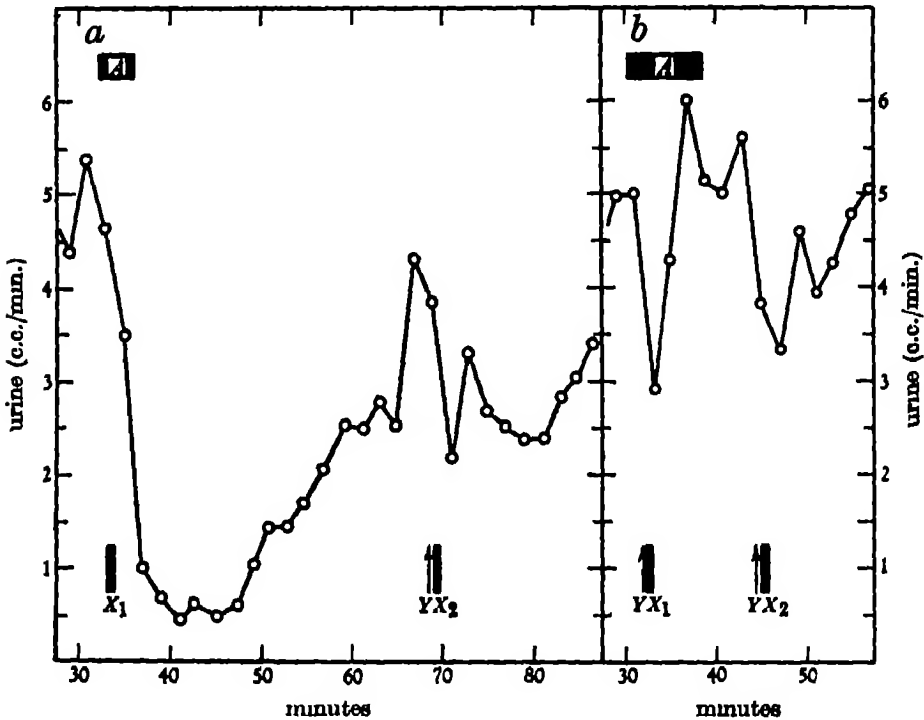
FIGURE 7. 'Nicky', in Pavlov stand. a, 13 November 1946; b, 18 November 1946. A, periods of occlusion of both common carotid arteries. At X_1 and X_2 three 1 sec. faradic stimuli followed by 30 sec. stimulus of weaker intensity. At Y, 5 μg. adrenaline intravenously. In experiment b the stimulus at X_1 and at X_2 was a little stronger than in experiment a. In experiment a the water loads at X_1 and X_2 were 279 and 217 c.c., and in experiment b 253 and 198 c.c. respectively. Abscissae: time after the test dose of water. The experiments show that occlusion of both common carotid arteries does not prevent the suppression by adrenaline of the emotional release of post-pituitary antidiuretic substance.

both carotids were occluded. Occlusion of the carotids has not interfered with the inhibitory effect of the faradic stimulus, a result which has been confirmed in each of four similarly conducted experiments. When, however, 5 μg. adrenaline are injected intravenously 30 sec. before the application of the faradic stimulus, the inhibitory effect of the stimulus is suppressed. This is shown in the two experiments of figure 6b. The stimulus (X) was a little greater than that given in the experiment B of figure 6a, and while in the one experiment (A, figure 6b) the inhibitory effect

of the stimulus is completely suppressed, in the other (B, figure 6b) a very small inhibition of pituitary origin is seen to follow the transitory effect of the adrenaline. Blood-pressure measurements made during the course of these two experiments are recorded at the top of the figure. In experiment A, the blood pressure following the injection of 5 μg. adrenaline at Y and during the period of stimulus X, did not rise above 150 mm. Hg, and after repetition of the injection at P the blood pressure did not reach 160 mm.; nor following similar injections at Q (experiment B) did it reach 165 mm. Hg. The results of a similar comparison between the effect of a stimulus during carotid occlusion and that following adrenaline injection, are given in figure 7a, the comparison here being made during the course of a single experiment. While carotid occlusion (A) has no appreciable influence on the inhibitory effect of the faradic stimulus (X), adrenaline (5 μg. at Y) almost abolishes the inhibitory effect of the subsequent stimulus (X), and this in spite of a much smaller water load than that subsisting at the time of the former stimulus. This effect of adrenaline is not appreciably altered when the injection and subsequent stimulus are given while the carotid arteries are being occluded (figure 7b).

These experiments show that adrenaline blocks the emotional release of post-pituitary antidiuretic substance by some process other than a rise in arterial pressure outside the territories supplied by the common carotid arteries (figure 6), and other than a rise in arterial pressure inside the territory supplied by the external carotid arteries (figure 7b). Moreover, seeing that, in the dog, there are arterial anastomoses between the ophthalmic branch of the internal carotid on the one hand, and the internal maxillary branch of the external carotid on the other (see p. 72), it would seem extremely unlikely that in the experiment depicted in figure 7b the carotid-sinus pressure was raised by the adrenaline injection to a normal, still more to a supernormal value. The evidence, then, is against the adrenaline-block's originating from a rise in sinus pressure. Two alternative modes of action of adrenaline in this regard remain for consideration, viz. through an increase in cerebral blood flow, and through a specific interference in the chain of chemical reactions initiated in the nervous system by the faradic stimulus and ending in the release of post-pituitary antidiuretic substance. While the experiments so far described afford no basis for distinguishing with certainty between these two possible modes, the results depicted in figure 7b are in favour of the latter alternative. If it were found that adrenaline was more effective in suppressing the inhibition of water diuresis by a faradic stimulus when infused into the carotids than when infused into a systemic vein, and that administration of the drug by the intracarotid route produced no greater rise in blood pressure than that by the intravenous route, the conclusion would be justified that adrenaline was blocking the release of antidiuretic substance by virtue of its molecular configuration rather than by virtue of an induced increase in cerebral blood flow. Experiments calculated to give an answer to this question have not yet been undertaken.

III. The release of post-pituitary antidiuretic substance by an increase in the osmotic pressure of the carotid blood

The demonstration in the normal animal of the release of pituitary antidiuretic substance by the artificial means of faradic stimulation of sensory nerves and receptors, and the fact that this release may be caused by such mild disturbance of the central nervous system as comes within a physiological range—even the anticipation of a nocuous stimulus which the animal has previously experienced may be effective—encouraged an attempt to determine whether the secretion of this substance were not continuously varying with, and under the direct control of, some factor in the animal's *internal* environment, to the maintenance of which factor within a narrow physiological range the renal secretion of water and of chloride would specifically contribute. Such factor is clearly the osmotic pressure of the arterial blood. Indeed, the hypothesis that water diuresis is conditioned by an inhibition of secretion of antidiuretic substance by the neurohypophysis implies a prior governance of this secretion by the osmotic pressure of the arterial blood. It was of interest, therefore, to determine the effects of an increase in this pressure on the secretion of the kidney during water diuresis.

A. *Methods*

(1) *Perineotomy and the formation of carotid loops*

Fully grown and healthy bitches varying in weight between 11 and 21 kg. have been used. They were perineotomized in order to make catheterization of the bladder simple, and after healing was complete a 'carotid loop' (Van Leersum 1911) was made on one side, followed after a suitable interval of time by the formation of a similar loop on the other side, the carotid sinus being sometimes denervated, sometimes not. In the formation of the loop, two parallel incisions were made through the skin and along the course of the common carotid, the artery was enfolded within the strip of skin by the suturing of its edges behind the vessel, and finally the outer edges of the skin incisions were approximated by a row of sutures deep to the tunnel of skin through which the carotid artery was now passing. Horsehair was used as suture material. Photographs of animals each with two such carotid loops are given in figures 12 and 13, plate 10.

(2) *Removal of the posterior lobe of the pituitary*

This was effected through the mouth, Aschner's (1912) diasphenoid route being adopted, and the technique being that described by Pickford (1939) with the exception that a tracheal cannula was not inserted, and that an electrically operated dental drill was used to remove the inner table of the sphenoid.

(3) *Removal of the thoracic and abdominal sympathetic chains*

The surgical procedure was essentially that first used by Cannon, Newton, Bright, Menkin & Moore (1929), and later recorded more fully by Bacq, Brouha & Heymans

(1934), the technique which was adopted being that described by Verney & Vogt (1938). The thoracic chains were removed in two stages, the abdominal in one. Removal of a thoracic chain included that of the stellate ganglion above and resection of the thoracic roots of the splanchnic nerves below; and removal of an abdominal chain included, unless otherwise stated, that of ganglia L1 above and S1 below, and resection of the abdominal part of the splanchnic nerves. The terms 'thoracic sympathectomy' and 'abdominal sympathectomy' will be used to connote the removals here described.

Other operative procedures occasionally used in this work will be described in their appropriate contexts. All were performed with full asepsis precautions and—with the exception of removal of the posterior lobe of the pituitary, where pentobarbitone sodium was given intravenously in 5 % aqueous solution in a dose of 33 mg./kg. body wt.—under ether anaesthesia. Recovery was rapid and uneventful.

(4) *Recording of the mean arterial pressure*

The trained animal lay on its side on a warmed table; and after a small branch of the femoral artery, e.g. the saphenous, had been located by palpation, the overlying skin was anaesthetized by the subcutaneous injection of 1 % procaine HCl, the vessel exposed with asepsis precautions, and into it was tied a blunted serum needle filled with a sterile solution of heparin (1 mg./c.c. 0·9 % NaCl) and connected, by pressure tubing containing sterile 0·9 % NaCl, with a mercury manometer recording on a smoked kymograph drum. On withdrawal of the serum needle the vessel was tied, and the skin incision was closed with interrupted silk sutures and protected with a collodion dressing.

(5) *Measurement of the common carotid blood flow in the anaesthetized dog*

Under chloralose anaesthesia (0·1 g./kg. body wt. intravenously) one common carotid artery was exposed; and after heparin (8 mg./kg. body wt.) had been injected intravenously, a modification of Stolnikow's (1886) stromuhr was interpolated between the ends of the divided carotid artery. The stromuhr (figure 8) was encased in a glass water-bath the temperature of which was maintained at 40° C, and the times of passage of the blood meniscus between two marks on each of the calibrated cylinders of the stromuhr were recorded by signal on a moving drum. A continuous record of the arterial pressure was obtained by mercury manometer connected with a cannula in one of the femoral arteries.

(6) *Intra-arterial infusion of gelatin masses*

In order to obtain a picture of the vascular bed of each carotid artery the following procedure was adopted. Carmine-gelatin and prussian blue-gelatin masses were prepared according to Gross's (1921) method, their relative viscosities, kindly determined for me by Dr Karvonen, being 30·4 and 28·6 respectively (temp. 38·7° C, water = 1). The apparatus used for their infusion is shown diagrammatically in figure 9. When this had been prepared, the temperature of the
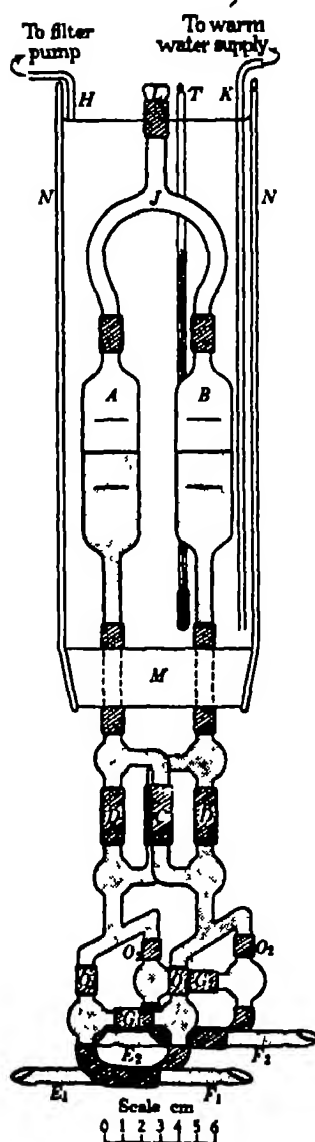
FIGURE 8 Diagram of modified Stolnikow-stromuhr. *N*, glass cylinder closed below by the rubber bung *M*. *A* and *B*, glass bulbs, each calibrated between the two marks. E_1E_2, cannulae in central ends, and F_1F_2, those in peripheral ends of the divided carotid arteries. The apparatus is filled with blood up to the halfway level of the bulbs, the upper half of the bulbs and the connecting tube *J* being then filled with liquid paraffin. *L*, stopper. *T*, thermometer. *K*, inlet of warm water from a constant-pressure reservoir, the water passing thence through a metal coil heated by a gas flame. *H*, water-outlet tube connected with a filter pump. The clamping of the tubes O_1 and G_2 puts the stromuhr into the right carotid circuit, that of tubes O_2 and G_1 into the left. When the tubes *C* are clamped the blood meniscus rises in bulb *B*, and when *D* are clamped it rises in bulb *A*. The apparatus is rigidly fixed to a 'Palmer' large adjustable screw-stand which allows the instrument to be moved easily in a vertical direction.

water-bath being 40° C, the animal was anaesthetized with chloralose, a tracheal cannula inserted, and both carotid arteries were exposed. Under artificial respiration the thorax was then opened, the pericardium incised, and two loose ligatures were placed around the superior vena cava. If the vertebral arteries were to be perfused as well as the carotids—in which event the reservoir E contained a plain-gelatin mass—the subclavian arteries were ligated just beyond the origins of the vertebrals,
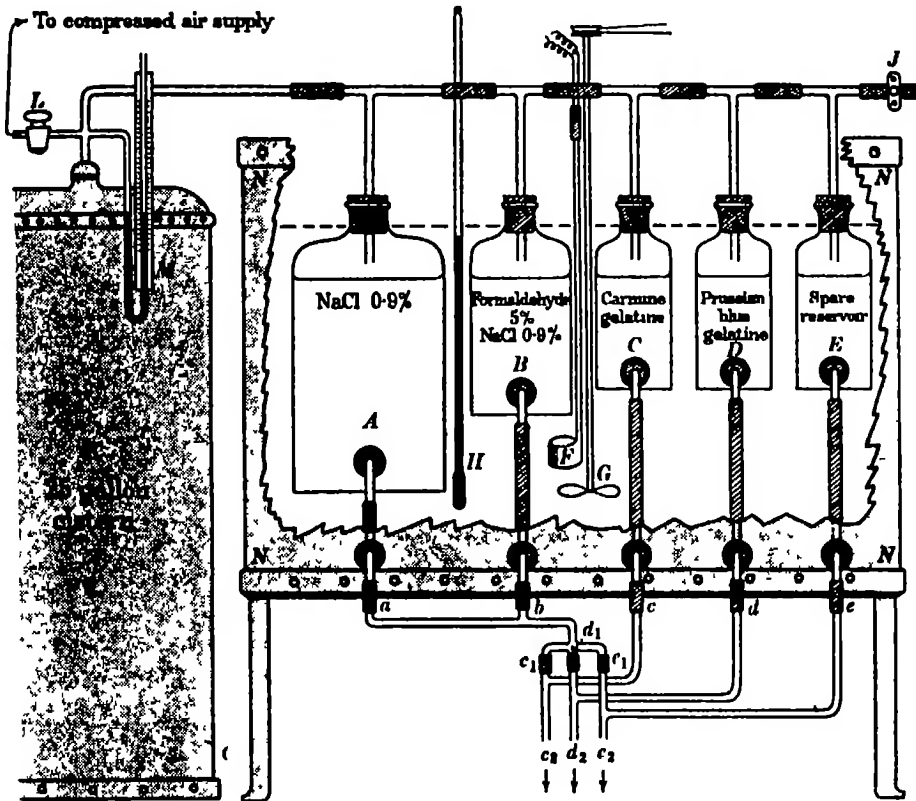
FIGURE 9. Diagram of apparatus for fixation of the head tissues. N, 60 l. galvanized iron water-bath. A to E, glass reservoirs. Capacity of $A = 3$ l., of $B = 1\cdot2$ l., of C, D and $E = 0\cdot6$ l. each. F, hot point. G, stirrer. H, thermometer. J, screw clip. K, galvanized iron air-reservoir, connected via the tap L with the compressed air supply. M, mercury manometer. c_2 and d_2, tubes leading to cannulae in the common carotid arteries. e_2, tube leading, when so desired, to a cannula in the arch of the aorta for perfusion of the vertebral arteries.

and the arch of the aorta was freed immediately distal to the origin of the left subclavian artery. When all the tubes outside the water-bath—they were heavily lagged with cotton-wool to prevent local cooling—had been filled to the cannulae tips with warm saline from the reservoir A, clamps were applied at a, b, c, d and e, the pressure in the air reservoir was raised to 120 mm. Hg, and the cannulae attached to c_2 and d_2 were tied into the carotid arteries. The heart was now clamped at the auriculoventricular junction, the aortic cannula rapidly inserted,

a cannula tied into the superior vena cava to drain away its effluent, and the clamp at *a* immediately released. When the effluent from the cava was fairly free from blood, the clamp at *a* was replaced and that at *b* removed; and after about 1 l. of the formaldehyde-saline had passed through the head tissues, the supply was switched off and the saline solution (about 1 l.) again infused. Clamps were then applied at *a*, c_1, d_1 and e_1, those at *c*, *d* and *e* released, and the pressure in the air reservoir was raised to 180 mm. Hg. The gelatin masses were now being infused, and after a suitable interval of time the tubes at *c*, *d* and *e* were *simultaneously* clamped. When the gelatin had set in the cannulae, the head of the animal was removed and stored in a refrigerator pending dissection.

(7) *Fixation of the head tissues at room temperature*

Two pairs of Marriotte bottles (3 l. capacity) were fixed 170 cm. above the head of the animal, each pair being connected via a T-piece and wide-bore rubber tube with a cannula for insertion into a carotid artery. One of each pair contained 0·9 % NaCl, the other 5 % formaldehyde in 0·9 % NaCl. Two pairs of bottles were used so that, if so required, the strength of sodium chloride solution in one pair could be different from that in the other. The head of pressure was equivalent to a perfusion pressure of 126 mm. Hg. The animal was anaesthetized with chloralose, both carotid arteries and external jugular veins were exposed, the carotid cannulae inserted, and cannulae tied into the external jugular veins to drain away their effluents. The infusion of the sodium chloride solutions was then immediately started, and the heart pierced through the chest wall. When the effluents were fairly free from blood, the sodium chloride solutions were switched off and the formaldehyde solutions on, and after about a litre of each of these had been infused, the carotids were tied above the cannulae. The head was then removed and stored either in a refrigerator or in a bath of 5 % formaldehyde in 0·9 % NaCl. Sometimes, instead of formaldehyde-saline, a mixture of 50 c.c. 40 % formaldehyde and 50 c.c. glacial acetic acid, made up to 1 l. with 80 % ethyl alcohol, has been used.

(8) *Reconstruction of anterior hypothalamic region*

In order to obtain a picture of the relations and extent of the supraoptic nucleus in the dog, a model has been made of the relevant structures in one small animal. Serial sections (10 μ) of the formaldehyde-fixed and paraffin-embedded material were cut in the frontal plane, stained with haemotoxylin and eosin, and a drawing of each was made on white cardboard (0·45 mm. thick) at a magnification × 50 by means of a Leitz Zeichenocular. The areas of the optic nerves, chiasma and tracts, of the supraoptic nuclei and of the corresponding part of the base of the brain were then cut away from the cardboard sheets and stuck together so as to form an enlarged model of the whole of this region. Since the model was made from paraffin- and not celloidin-embedded material, it was not an exact reproduction, but it was sufficiently accurate to give in one view a reliable picture of the relations and extent of the supraoptic nuclei. Moreover, in the serial drawings the blood

supplies to the anterior and posterior divisions of the nuclei could readily be followed to their source. A photograph of the model is given in figure 10, plate 9.

(9) *Determination of the sectional area of the lumen of the common and of the internal carotid artery*

Projection drawings at a magnification × 50 were made of transverse sections of the formaldehyde-fixed vessels. The circumference of the lumen was measured with a curvimeter and the area of the lumen computed.

(10) *Histological*

Sections of fixed and paraffin-embedded material were cut at 10 μ thickness, and stained with haematoxylin and eosin. For the identification of fat, two methods were used. In the one, small pieces of formaldehyde-fixed tissue were soaked in gum, frozen sections cut and stained with Scharlach R; in the other, the tissue was fixed in Flemming's fluid, embedded in paraffin, and serial sections were cut at 10 μ thickness.

(11) *Determination of the water content of blood*

The percentage (v/v) of water in the arterial blood during water diuresis was determined by withdrawing 11 c.c. of blood from a carotid into an all-glass syringe moistened (0·1 c.c.) with a solution of heparin (2 mg./c.c. 0·9 % NaCl), ejecting the contents into a tared 10 c.c. sp.gr. bottle, weighing, transferring about 1 c.c. to a tared and stoppered weighing bottle, weighing, and finally drying the blood to near constancy in weight in a phosphorus pentoxide desiccator (under graded reductions of pressure) in a refrigerator. The percentage (w/w) was determined from the loss in weight of about 1 c.c. of blood when dried by the same means. Here, however, each blood sample was collected in a dry syringe and immediately ejected into a tared weighing bottle.

(12) *Determination of blood chloride*

The method of Van Slyke & Sendroy (1923)—the silver nitrate solution and the nitric acid being added separately to the blood, as recommended by Wilson & Ball (1928)—was used, and the tubes were cooled in water and ice immediately before the titration. Duplicate estimations were made on 1 or 2 c.c. samples of blood, the blood being drawn into 2·5 or 5 c.c. all-glass syringes either containing potassium oxalate (3 to 6 mg./c.c. blood) or moistened with a saturated solution of the salt, in the latter instance a small and previously determined correction being applied for the dilution so occasioned. When the time course of the blood chloride during infusions of sodium chloride solutions was being determined, the latter method of preventing coagulation was adopted, the same syringe being used throughout and washed with distilled water after each blood sample had been obtained. The time and period of collection of each sample were signalled on a rapidly moving drum.

(13) *Determination of urine chloride*

With the omission of the digestion procedure the method was the same as for blood, the chloride being estimated in 2 c.c. samples. In those instances in which the chloride concentrations of the urine are plotted graphically, each value is antedated by the period of collection of the preceding $x + 1$ c.c. of urine, where x is the volume of the catheter and its extension tube, the 1 c.c. being added for the dead space of the urinary tracts. The volume flow and the chloride concentration of the urine are thus brought into more accurate temporal accord.

(14) *Determination of the post-pituitary extract equivalents of the inhibitions of urine flow*

The inhibitory responses to intracarotid injections and infusions of hypertonic solutions during water diuresis were assayed in terms of post-pituitary extract by equating these responses with those produced by suitable amounts of the extract given intravenously under the same conditions. The extract used was that marketed by Messrs Burroughs Wellcome under the name 'Infundin' and standardized to contain 10 i.u /c.c. The same batch of extract was used throughout so far as possible. If a change were necessary, the antidiuretic activity of the new batch was carefully compared with that of the old: no differences in activity were detected. The extract was suitably diluted with sterile 0·85 % NaCl; and when necessary for purposes of control, the penultimate and final dilutions were made with the particular solution the effects of whose intracarotid administration were the object of assay. The terms milliunit (mU) and microunit (μU) will be used to denote the antidiuretic activity of 10^{-4} and 10^{-7} c.c. respectively of the commercial extract.

(15) *The care and training of the animals, their preliminary treatment, and the circumstances of the experimental procedures*

Each bitch was given a diet of minced horse-flesh 120 g., biscuit 180 to 300 g. according to the size of the animal, and cod-liver oil 5 c.c. It was fed each evening at 5 p.m , and drinking water was always in the kennel except after the administration of the preliminary ('hydrating') dose of water. Comparable experiments were as a rule done at the same time of day; and when not otherwise employed during the daytime, the animals were in the open air on the flat roof of the building.

They were carefully trained to lie quietly on their right side for long periods, and all observations were made in the special chamber to which I have earlier referred. The temperature of this room was only roughly controlled, and lay between the limits of 19·5 and 22° C during the periods of these observations.

A preliminary dose of warm tap water was given by stomach tube, the animal was then returned to its kennel, and 1½ to 2 hours later it was brought to the experimental room, placed in a Pavlov stand, catheterized, given the test dose of water—300 to 400 c.c. (temp. 37° C), according to size of animal—and then laid on its right side on a warmed table, the urine thereafter being collected continuously into a series of graduated glass tubes. In a series of cognate observations on any

one animal each of the preliminary and each of the test doses of water was the same, and the periods between the two doses did not vary by more than 5 min. Before an intravascular injection the local area of skin was shaved and cleaned with surgical spirit.

(16) *Intracarotid and intravenous injections and infusions*

The preparation of the solutions. The solutions were carefully prepared from analytical reagents which, except in the earlier observations, had been dried over phosphorus pentoxide in vacuum desiccators. Triple glass-distilled water was used, and after all insoluble contaminants had been removed from the solutions by repeated filtration, the solutions were sterilized by heat.

Intracarotid and intravenous injections: short period (5 to 20 sec.). For the intracarotid injections a smoothly working syringe of 2, 5, 10 or 20 c c capacity was selected, and a stainless steel injection needle was attached thereto by means of a rubber tube (external and internal diameters 4 and 2·5 mm. respectively) some 15 cm. long, into which had been interpolated near the needle a short piece of glass tubing whereby the entry of the needle into the lumen of the vessel was confirmed. The needles usually employed had an external diameter of 0·7 mm., and each was selected after careful examination of its point by means of a lens. They were kept in absolute alcohol, and the rest of the injection apparatus was sterilized in boiling water immediately before the experiment. The head of the animal was supported in a convenient and comfortable position by a woollen pad covered by a clean towel; and after the syringe and its appendage had been filled with the solution, previously warmed to body temperature, and 0·1 c.c. sterile 0·85 % NaCl drawn into the needle, the carotid loop was taken between finger and thumb, and the needle gently pushed through the arterial wall and some centimetre or so along the lumen in the direction of the blood stream. The injection was then made as uniformly as possible with the aid of a metronome beating seconds. The carotid loop was left quite free during and for about 2 min. after the actual injection, and on withdrawal of the needle the site of puncture was slightly compressed for a minute or so, but not to such degree as seriously to impede the flow of blood within the vessel. The puncture of skin and vessel was apparently painless, and the animal was quite undisturbed by the injection, although often its appreciation of the salinity of the injected fluid was registered by a smacking of the lips towards the end of, and immediately after the injection. With the more concentrated solutions of sodium chloride and rapid injections, however, manifestations of cortical stimulation might ensue; these were avoided so far as possible in order to ensure that the urine-flow responses were causally uncomplicated by an emotional component. In any one animal injections have been made at approximately the same time after the administration of the test dose of water, and all, unless otherwise stated, have been made without technical hitch. Intravenous injections, to control the effects of the intracarotid injections, were made under the same conditions into the left malleolar vein—on rare occasions, into the external jugular vein.

Intracarotid and intravenous infusions: long period (10 *and* 40 *min.*). These were effected by means of a constant-speed motor connected by reduction gearing with the head of a micrometer screw which moved forward the plunger of the syringe with which the fine infusing needle was connected. In these instances the needle had an external diameter of 0·4 mm. The apparatus and its arrangement for such infusions are shown in figure 11, plate 9. The needle having been introduced into the vessel, the lumen of the needle is maintained free from blood by the slow compression of the plunger of syringe *G*, containing NaCl 0·85 %. At an appropriate moment the contents of syringe *H* are emptied into syringe *G* so as to wash the physiological saline from the environment of the needle, the clamps *J* and *K* rapidly closed, and the motor *A* is immediately started. The test solution is now being infused at a constant rate. All the solution-containing apparatus was sterilized in boiling water immediately before an experiment. The reduction gearing and throw of the micrometer were such that infusions could be made for periods of 10 or 40 min.

(17) *Estimation of the volume flow of blood in the common carotid*

Tschuewsky (1903), by means of Hurthle's (1903) registering stromuhr, measured the carotid flow in dogs under the influence of morphine and deeply anaesthetized with chloroform and ether, and in nine animals whose weights lay between 11·2 and 19·7 kg. (mean = 14·1) the average flow was 0·138 c.c./kg./sec., and blood pressure 76 mm. Hg. The values found by Rein (1929*b*) in six animals whose weights lay between 4 and 13 kg. (mean = 8·3) gave an average of 0·143 c.c./kg./sec.: the animals were under the influence of morphine and were lightly anaesthetized with chloralose, their average blood pressure being 99 mm. Hg. Using the modification of the Stolnikow stromuhr described above, I have measured the carotid flow in three animals anaesthetized with chloralose, and the results are given in table 1. From these figures and those given by Tschuewsky (1903) and by Rein (1929*b*), we see that the carotid flow in the anaesthetized dog is of the order of 0·13 c.c./kg./sec.

TABLE 1. COMMON CAROTID BLOOD FLOW IN DOGS ANAESTHETIZED WITH CHLORALOSE

body wt. (kg.)	blood pressure (femoral artery) (mm. Hg)		carotid flow (c.c./sec.)			
			right		left	
12·6	132	—	2·20	—	—	—
10·5	141	**149**	1·04	**1·36**	—	—
	142	**152**	—	—	1·08	**1·32**
*19·7	134	**148**	1·53	**2·10**	—	—
	'131	**131**	—	—	1·60	**2·12**

* Right carotid sinus denervated. The figures in heavy type are the pressures and flows when the contralateral carotid was occluded. The drop in pressure across the stromuhr at blood flows in the above range was 5 mm. Hg.

It was, however, highly desirable to obtain a measure of this flow under living conditions, and in the individuals in which the responses to intracarotid injections and infusions were being determined. Attempts to do so have not been very successful. Two methods were tried. In the first, NaCl 4·7 M was infused into one of 'Nicky's' (see p. 56) carotids at 1·0 c.c./min. over a period of 10 min., the infusion needle being introduced as low down in the loop as possible; and blood samples were taken from the upper part of the same vessel and from the contralateral carotid, and their chloride concentrations determined. Calculation from the time courses (see figure 32) of the chloride concentrations in the two sets of blood samples gave inconsistent values for blood flow, e.g. in one experiment they ranged

TABLE 2. CAROTID BLOOD FLOWS IN THE LIVING DOG,
BY THE EVANS'S BLUE METHOD

'Whitethroat' experiment	carotid flow (c.c./sec.) right	carotid flow (c.c./sec.) left	'Nicky' experiment	carotid flow (c.c./sec.) right	carotid flow (c.c./sec.) left
1	—	1·7	1	—	2·1
	—	2·1		—	3·2
	—	1·6		—	1·8
2	1·9	—	2	2·7	—
	1·7	—		3·0	—
3	—	2·3		1·7	—
	—	2·1		1·6	—
	—	*1·5	3	—	1·8
	—	2·2		—	1·8
4	2·4	—		—	3·6
	*1·2	—		—	1·5
			4	1·2	—
				1·2	—
				1·6	—
				1·4	—
Averages	1·8	1·9	Averages	1·8	2·3

* Restlessness just before the blood sampling.

between 2 and 7 c.c./sec. (seven observations, mean = 3·5 c.c./sec.). The points of the infusion and sampling needles were about 5 cm. apart; and inconstancy in the rate of withdrawal of a sample, and incomplete mixing of the infused solution with the blood in the time available (a mean of probably less than 250 msec.) are seemingly the chief sources of the inconsistency of the values. In the second method, a solution of Evans's blue was infused for 40 min. into a carotid at a rate of 12·7 or 16·7 μg./sec., and a series of blood samples (3 c.c. by syringe moistened with heparin, 1 mg./c.c. 0·9% NaCl) was taken from the ipselateral ear vein and from the malleolar vein. From the haematocrit values and the concentrations of Evans's blue (method of Crooke & Morris 1942) in the plasma—I am indebted to Dr Karvonen for these determinations—the common carotid blood flow was calculated.

Occasional sampling from the marginal vein of the homolateral ear showed that the concentration of Evans's blue in this plasma lay on the linear slope of the concentrations in the systemic plasma. No blood, therefore, was reaching the ear from the contralateral carotid; and carmine- and prussian blue-gelatin infusions have confirmed this, and have indicated, furthermore, that the vertebral arteries do not contribute to the auricular supply. Four experiments were made on each of the two dogs 'Whitethroat' (see p. 56) and 'Nicky'. The results are given in table 2. The variations are wide, but are less than those encountered with the former method. As there were difficulties with the technique, and the animals were sometimes disturbed by the manipulations ('Nicky' had then been 'sympathec-tomized', but the cervical part of the sympathetic system, craniad to the stellate ganglion, was intact), a probable significance is to be attached to the average values only.

While the results of these attempts to obtain a measure of the carotid flow in the living animal are far from satisfactory, they are of the same order as those obtained in anaesthetized animals—the average of Tschuewsky's (1903), Rein's (1929b) and my own figures is 0·13 c.c./kg./sec., and with 'Nicky', on whom most of the experiments to be described later have been made, I have taken 2·2 c.c./sec. as the volume flow through each of her carotids. It is improbable that this is an over-estimate. With the other animals, too, I have assumed a carotid flow of 0·13 c.c./kg./sec.

(18) *Calculation of the local change in osmotic pressure produced by the intracarotid injections and infusions*

The needles used for the injections had a sectional area of 0·38 mm.². Now Tschuewsky (1903) obtained the mean diameter of the lumen of the carotid in the anaesthetized dog from measurement of the maximum and minimum external diameters during the passage of the pulse wave, and the thickness of the walls when the vessel was occluded. The measurements were made on nine animals whose weights lay between 11·2 and 19·7 kg. (mean = 14·1), and the diameters of the lumina were between 2·80 and 3·80 mm. (mean = 3·25). I have made similar measurements in two animals whose weights were 10·5 and 19·7 kg., and the dia-meters of the lumina were 3·0 and 3·6 mm. respectively. The needles, then, produce a diminution of some 4 % in the sectional area of the lumen, and I have neglected the effect of this on the volume flow through the carotids. The smaller needles used for the infusions had a sectional area of 0·12 mm. only.

I have assumed that the injections and infusions do not change the volume flow through the carotid, and that osmotic equilibrium is attained by the time the blood reaches the receptors from which the response of the neurohypophysis is initiated. With respect to the former assumption, any possible change in the volume flow would, in the case of the long-period infusions and the majority of the short-period injections, be very small; and seeing that the proof of the osmotic determination of the response of the neurohypophysis derives, as will be shown later, from the

injection or infusion of the *same* volumes of osmotically comparable solutions in the *same* times, and that when the rate of a short-period injection is high (e.g. 1 c.c./sec.), the response is as would be expected on the basis of this assumption, the assumption is a valid one. The evidence for the validity of the second assumption is as follows. The receptors, as will be shown later, lie in the vascular bed normally supplied by the internal carotid artery. Now if, as seems reasonable, these receptors are reached at a time which is not sensibly different from that at which the retina is reached, the following observations are relevant. In a chloralosed and atropinized dog I watched with an ophthalmoscope the fundus of the left eye when 1 c.c. 1 % Evans's blue was suddenly injected into the left common carotid artery. A transitory blue flush was seen in the non-pigmented area of the fundus, and the interval between the injection and the flush was 2·7 sec. (mean of four observations: extremes 2·0 and 3·0 sec.). When the injection was made into the right carotid no flush appeared in the left retina. The left carotid was then occluded—this caused no change in the appearance of the retinal vessels—and the injection into the right carotid was repeated: a blue flush was seen in the left retina, but it was much paler than when the injections had been made into the left carotid. The time of its appearance, however, was not appreciably different (two observations, 2·9 and 2·8 sec.). In a second experiment, similarly conducted, the results shown in table 3 were obtained. The observations were made immediately after the measurements of the carotid blood flow given in table 1, and the average blood pressure during the observations was 114 mm. Hg. The animal weighed 19·7 kg., and the right carotid sinus had been denervated. No change was detected in the appearance of the retinal vessels when either or both common carotids were occluded. In both of the foregoing experiments the tip of the injection needle was at about the same place in the carotid as in those experiments on the living animal in which carotid loops had been made: in the second experiment the distance between the needle tip and the carotid sinus was 8 cm.

TABLE 3. TIMES OF APPEARANCE OF EVANS'S BLUE IN THE RETINAL VESSELS WHEN THE DYE IS INJECTED INTO A CAROTID ARTERY. DOG, WT. 19·7 KG. CHLORALOSE ANAESTHESIA

retina observed	carotid injected	time between injection and blue flush (sec.)			remarks
		extremes	number of observations	mean	
left	left	1·8, 2·7	3	2·2	
left	left	1·4, 1·6	2	1·5	right carotid occluded
left	right	3·0, 3·4	3	*3·1	left carotid occluded
right	right	2·5, 3·0	5	2·6	
right	right	1·5, 1·9	2	1·7	left carotid occluded
right	left	2·9, 3·0	2	*2·9	right carotid occluded

* Retinal flush paler than in the other instances.

In the chloralosed dog, therefore, the time of blood transit from about the middle of the common carotid to the retina is some 2·5 sec. It was of importance to obtain, if possible, a measure of this time in the living animal, and the results of such an attempt on 'Nicky' are given in table 4. Each pupil was dilated by putting into the conjunctival sac a lamella of homatropine (0·65 mg.) and one of cocaine HCl (1·3 mg.), and the conditions and circumstances of the observations were the same as those under which intracarotid and intravenous injections and infusions were regularly given. As with the anaesthetized dogs, occlusion of one or both common carotid arteries caused no appreciable change in the appearance of the retinal vessels, and this has been confirmed in another animal ('Whippet', number 295, p. 56). When the dye was injected the retinal background did not, as in the anaesthetized animals, become suffused with blue, but the vessels appeared momentarily black.

TABLE 4. 'NICKY'. TIMES OF APPEARANCE OF EVANS'S BLUE IN THE RETINAL VESSELS WHEN 0·8 C.C. OF A 1% SOLUTION IS INJECTED INTO A CAROTID ARTERY IN 1 SEC. TWO SERIES OF OBSERVATIONS

The means of the periods are printed in heavy type

retina observed	carotid injected	time between injection and appearance of dye in retina (sec.)	remarks
left	left	3·7, 3 5, 3 6	—
left	left	3·2, 3·4, 3·3	right carotid occluded
right	right	3·2, 2·6, 2 6, 2·8	—
left	left	3·1	—
right	left	∞	—
right	left	3 8	right carotid occluded
right	right	2 5	—
left	right	∞	—
left	right	3 8	left carotid occluded

These observations will be considered again later in connexion with the effects of ligation of the internal carotid on the response of the neurohypophysis to intracarotid injections of hypertonic solutions, and, in 'Nicky', with the quantitative difference between the response to an injection into the right carotid and that to the same injection into the left carotid. The pertinent point for the moment is that the period of passage of blood from about the middle of the common carotid to the retina—and that to the pituitary receptors is unlikely to be appreciably less— is some 2·5 sec., a period which, from the data of Jacobs (1932), is fully adequate for the attainment of osmotic equilibrium between the blood and the solutions injected or infused in the experiments to be described later.

With 'Nicky' the percentage (v/v) of water in the arterial blood during water diuresis was found to be 83·0, and I have used this figure with the other animals as well. From the molarity of the injected or infused solution and the molal volume

of the solute, the number of mols of solute and of c.c. of water added to the blood in a given time were derived; and from these figures and the volume of water in the blood passing the needle in the same time, the increase so produced in the osmolality of the carotid blood was calculated. The following are the molal volumes (c.c.) used in the calculations: NaCl 17, Na_2SO_4 23, dextrose 110, sucrose 210, and urea 45. The molal volume has been assumed not to change with changing molarity except in the case of sodium sulphate, and here the molal volume at different molarities has been calculated by means of the formula given by Harned & Owen (1943): molal volume $= 11.47 + 12.16\sqrt{c}$, where c is the molar concentration. The molality of the arterial blood of the dog has been taken as equivalent to 0·150 molal NaCl, and 85 % as the apparent degree of dissociation of this sodium chloride ($= 0.277$ osmolal). The apparent degree of dissociation refers to measurements of the freezing-point. Since the maximum increase produced in this molality by the injections has been of the order of 100 % only, the apparent degree of dissociation has been assumed constant. With the injections of sodium sulphate, the calculated final molality in the blood has been between 0·08 and 0·11, and with the 10 min. infusions 0·006; with the former the apparent degree of dissociation of the salt has been taken to be 66 %, and with the latter 72 %. In the results which follow, the calculated increases in the osmolality of the carotid blood produced by the injections and infusions have been expressed as percentage increases in osmotic pressure.

B. *The animals and their operation histories*

The following animals have been used in this investigation, and the relevant operative procedures to which they were subjected are here given in brief:

'Alice', number 307. Wt. 15 kg. 8 March 1943. perineotomy. 9 April 1943: carotid loop made on left side, the sinus *not* being denervated. 6 May 1943· carotid loop made on the right side, the sinus *not* being denervated. 21 September 1943: Hering's nerve divided on left side well above the carotid bifurcation. 11 November 1943: right and left splanchnic nerves divided at the crura of the diaphragm; suprarenal and abdominal sympathetic chain removed on right side. 29 November 1943: operation with a view to tying the left internal carotid artery. When the animal was killed (12 January 1944) it was found that the left internal carotid was patent, two smaller vessels having been tied instead.

'Sally', number 280. Wt. 21 kg. December 1940: perineotomy. 20 June 1941: carotid loop made on left side, the carotid sinus being denervated. 26 February 1943: fundus of bladder excised (see Rydin & Verney 1938), owing to periodic retention of urine in bladder during diuresis experiments. 5 March 1943: carotid loop made on right side, the carotid sinus *not* being denervated. 22 September 1943: bifurcation of left common carotid exposed with a view to removing the carotid body if it were present. Histological examination of the pieces removed revealed no carotid body tissue. 5 November 1943: attempt to tie left internal carotid artery. In being dissected from surrounding scar tissue it ruptured, and both

external and internal carotids were tied, and the common carotid was ligated just below its bifurcation. 6 December 1943: removal of posterior lobe of pituitary. Sections of the parts removed showed them to be mostly posterior lobe and pars intermedia; two small fragments were from the anterior lobe. 1 January 1944: right internal carotid divided between ligatures about 5 mm. from its origin. 26 January 1944: formaldehyde-fixation of head and neck tissues (cannula in aorta), followed by carmine-gelatin infusion into right common carotid. Histological examination of pituitary region showed that no pars nervosa or intermedia was present and that some of the pars distalis was missing.

'Nicky', number 309. Figure 12, plate 10. Wt. 17 kg. 22 March 1943: perineotomy. 6 April 1943: carotid loop made on left side, the sinus *not* being denervated. 14 June 1943: carotid loop made on right side, the sinus *not* being denervated. 29 March 1944: denervation of kidneys, section of right and left splanchnic nerves at the crura of the diaphragm, abdominal sympathectomy L 1 to L 5 inclusive. 18 May 1944: thoracic sympathectomy on right side. 26 May 1944: thoracic sympathectomy on left side.

'Jock', number 277. Wt 17 kg. 28 October 1940: perineotomy. 5 December 1941: carotid loop made on left side, the sinus being denervated. 15 April 1943: carotid loop made on right side, the sinus *not* being denervated. 17 May 1944: thoracic sympathectomy on right side. 2 June 1944: thoracic sympathectomy on left side. 24 March 1947: abdominal sympathectomy and removal of left suprarenal.

'Pat', number 308. Figure 13, plate 10. Wt. 15 kg. 8 March 1943: perineotomy. 2 June 1944: carotid loop made on left side, the sinus *not* being denervated. 12 January 1945: carotid loop made on right side, the sinus *not* being denervated. 23 February 1945: abdominal sympathectomy. 10 April 1945: thoracic sympathectomy on left side. 1 May 1945: thoracic sympathectomy on right side. 27 November 1945 left internal carotid divided between ligatures just beyond sinus, no nerve tissue being sectioned.

'Whisky', number 306. Wt. 17 kg. 8 April 1943 perineotomy. 20 April 1943: carotid loop made on left side, the sinus *not* being denervated. 26 May 1943: carotid loop made on right side, the sinus *not* being denervated.

'Julie', number 335. Wt. 11 kg. 20 September 1946: perineotomy. 1 January 1947: carotid loop made on right side, and the external carotid artery tied at its origin, the sinus *not* being denervated. 24 January 1947: carotid loop made on left side, the sinus *not* being denervated. 20 February 1947 resection of abdominal splanchnic nerves, and removal of sympathetic chains from L 1 to L 3.

'Whitethroat', number 303. Wt. 15 kg. 2 December 1942: perineotomy. 8 January 1943: carotid loop made on left side, the sinus being denervated. 30 April 1943: carotid loop made on right side, the sinus *not* being denervated.

'Whippet', number 295. Wt. 15 kg. December 1940: perineotomy. 21 May 1941: carotid loop made on left side, the sinus being denervated. 13 April 1943: carotid loop made on right side, the sinus *not* being denervated. 5 March 1947: formaldehyde-fixation of head and neck tissues (cannulae in carotids).

Other animals, eighteen in number, some of which had been used primarily for other investigations, have supplied material for anatomical and histological study. The head and neck tissues were fixed and, in some instances, perfused through the carotid and vertebral arteries with carmine-, prussian blue- and plain-gelatin masses. Any findings which bear on the experimental results will be given when these are described or discussed.

C. *Results*

(1) *Control observations*

On the effects of compression of the carotid. Since the technique of intracarotid injection was associated with temporary digital compression of the vessel, it was necessary to see whether this *per se* produced any change in urine flow during water diuresis, or any overlasting change in blood pressure. With 'Sally' it was found that occlusion of the right carotid (innervated sinus), of the left (denervated sinus) and of both for periods ranging between 30 and 120 sec. (twelve observations) were neither accompanied nor followed by any appreciable change in the course of the diuresis. Occlusion of the right carotid for 10 min., however, was associated with an increase in urine flow from 3·25 to 4·0 c.c./min., the increase beginning in
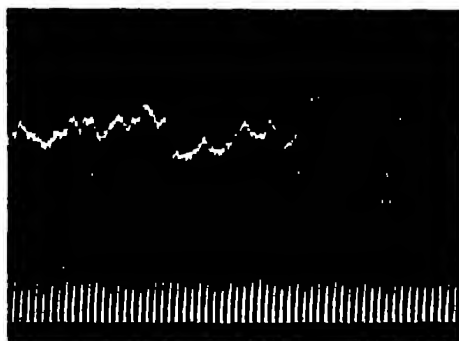
FIGURE 14. 'Sally'. 26 March 1943. Femoral arterial pressure record under local anaesthesia. Between *a* and *b* the left common carotid artery was occluded, and between *c* and *d* the right.

the second minute of occlusion and disappearing completely during the second to the fifth minute after arterial release. thereafter the urine flow followed its usual course. The systolic pressure, determined by means of a pneumatic cuff applied to the left carotid loop, was raised during the period of occlusion from 126 to 145 mm. Hg, but a record of the femoral arterial pressure under local anaesthesia (figure 14) showed that the rise in arterial pressure produced by occlusion of the right carotid had disappeared 10 sec. after release (this was confirmed with 'Alice'; see also figure 5). From these observations it was concluded that any effect on urine secretion which might be seen to follow intra-arterial injection, would not be owing to such transitory interference with the carotid blood flow as might occur during the technique of puncture, and that any such transitory change in blood flow would have disappeared by the time the actual injection was being given.

On intracarotid injections of isotonic solution of sodium chloride; on the temperature of the injected solution; and on intravenous injections of hypertonic solutions of sodium chloride. With 'Sally' it was found that the injection into the left carotid of 10 c.c. 0·140 M-NaCl in 25 sec., of 12 c.c. of the same solution in 10 sec., of 20 c.c. 0·144 M-NaCl in 20 sec. (two experiments), and of 20 c.c. of the same solution in 19 sec. had no influence on the course of water diuresis. In one of the last three experiments the temperature of the solution on injection was 20·5°, and in the other two it was 41°. This absence of effect of isotonic solution of sodium chloride when injected in these amounts and at these speeds and temperatures is illustrated in figure 15*a* and *b*.
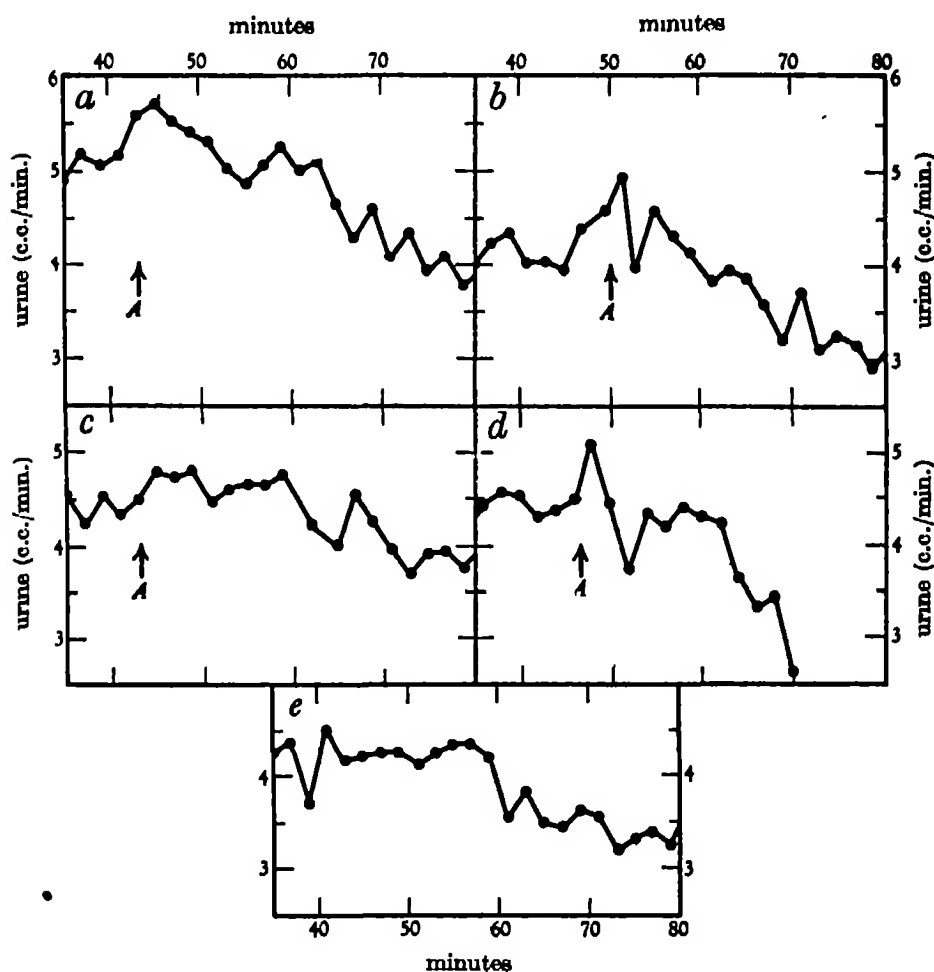
FIGURE 15. 'Sally'. Control observations on intracarotid and intravenous injections during water diuresis. *a*, at *A*, injection of 20 c.c. 0·144M-NaCl into left carotid in 20 sec., the temperature of the solution being 20·5° C, *b*, at *A*, injection of 20 c.c. 0·144M-NaCl into left carotid in 20 sec., the temperature of the solution being 41° C. *c*, at *A*, injection of 20 c.c. 0·343M-NaCl into left malleolar vein in 20 sec. *d*, at *A*, injection of 20 c.c. 0·855M-NaCl into left malleolar vein in 20 sec. *e*, diuresis curve, no injection being given. Abscissae: time after the test dose of water.

In subsequent experiments, therefore, the exact temperature of the injected solution was not determined, but it always lay well within the above range. The effects of intravenous injections of hypertonic solutions of sodium chloride were then tested, and it was found that there was no appreciable response to injections into the malleolar vein of 12 c.c. 0·343 M-NaCl in 60 sec., of 12 c.c. in 12 sec., of 20 c.c. in 20 sec., nor to an injection into the left external jugular vein of 12 c.c. 0·343 M-NaCl in 8 sec.; there was no immediate response even to the injection of 20 c.c. 0·855 M-NaCl into the malleolar vein in 20 sec., the only effect being an earlier subsidence of diuresis, doubtless owing to the temporary fixation of part of the water load as isotonic solution in the blood and tissues (figure 15c and d). When, however, hypertonic solutions of sodium chloride were injected into the carotid, definite inhibitory responses were observed. The effects of such injections have always been controlled by the injection of isotonic solution in the same volumes and at the same rates as with the hypertonic solutions.

(2) *The effects of hypertonic solutions of sodium chloride injected into the carotid artery*

The effects of such procedures in two animals are illustrated in figures 16 and 17. In figure 16 ('Alice') are shown (a) the response to the injection into the left carotid of 10·5 c.c. of 0·257 M-NaCl in 9 sec., and (b) that to the injection into the right carotid of 11 c.c. of the same solution in 13 sec. No effect was observed when 11 c.c. of 0·147 M-NaCl were injected in 9 sec. As may be seen, the hypertonic solutions produced an inhibition of urine flow from which there was gradual recovery towards the preinjection rate. Similar results were obtained from the other animals:
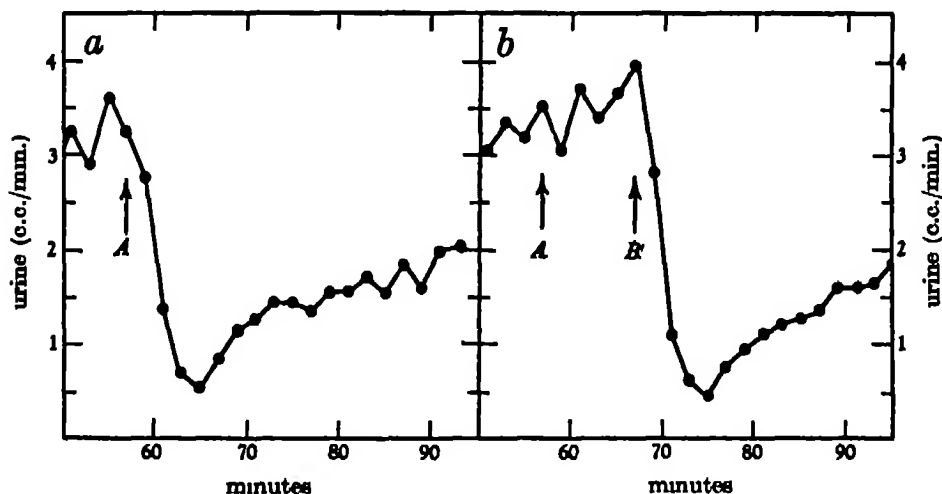
FIGURE 16. 'Alice'. a, 16 June 1943. At A, injection of 10·5 c.c. 0·257M-NaCl into left carotid in 9 sec. b, 17 June 1943. At A, injection of 2 c.c. 0·257M-NaCl into right carotid in 7 sec. and at B, injection of 11 c.c. of same solution into right carotid in 13 sec. Abscissae: time after the test dose of water.

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those from 'Sally' are illustrated in figure 17. It was found that the injection of 0·214 M-NaCl in amounts and at rates up to 11 c.c. in 11 sec. had no effect on the rate of urine secretion. Twenty c.c. of 0·257 M-NaCl injected in 16 sec. (graph a) and
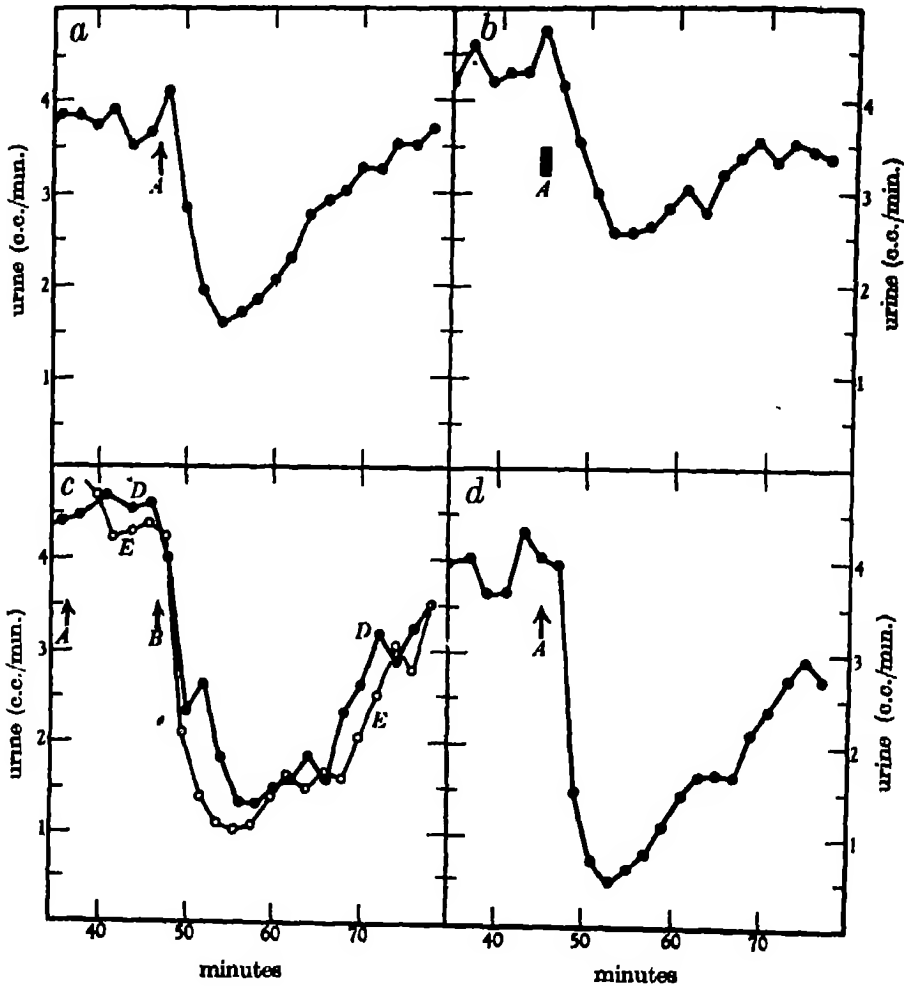
FIGURE 17. 'Sally'. a, 17 August 1943. At A, injection of 20 c.c. 0·257M-NaCl into left carotid in 16 sec. b, 10 June 1943. At A, injection of 12 c.c. 0·343M-NaCl into left carotid in 60 sec. c, 19 and 14 June 1943. At A, injection of 12 c.c. 0·139M-NaCl into left carotid in 10 sec.; and at B, injection of 12 c.c. 0·343M-NaCl into right carotid in 15 sec. (graph D), and in 10 sec. (graph E). d, 5 September 1943. At A, injection of 20 c.c. 0·343M-NaCl into left carotid in 25 sec. Abscissae: time after the test dose of water.

12 c.c. of 0·343 M injected in 60 sec. (graph b), however, produced small but quite definite inhibitions, and when the stronger solution was injected at greater rates (graphs c) and in larger amount (graph d) the inhibitory responses were increased. The magnitude of the response, therefore, varies with the strength of the solution

at constant rate and period of injection, and with the period of the injection at constant volume and strength of solution. The concentration of pigment and of chloride in the urine increased during the inhibition, and varied directly with its degree.

The character of these inhibitory responses simulated so closely the known character of the response to the intravenous injection of pituitary (posterior lobe) extract that it appeared highly probable that they were of pituitary origin. The test of this hypothesis was undertaken with 'Sally' in determining whether the response disappeared after removal of her posterior lobe.

(3) *The effect of removal of the posterior lobe of the pituitary on the response to intra-carotid injections of hypertonic solutions of sodium chloride*

Before the stage of removal was reached, however, the animal had been subjected to certain intervening procedures, and its sensitivity to injections which formerly were strongly effective became diminished. This diminution may well have been apparent in part, since the left common carotid had been ligated, and an increase in the rate of blood flow in the other would be expected to have occurred (see table 1, p. 50). Indeed, 26 days after the one carotid had been tied, and 5 days before removal of the posterior lobe, a note was made to the effect that the other 'felt bigger' than before. During the period between ligation of the left common carotid and removal of the posterior lobe, the results given in the upper part of table 5 were obtained. All the injections were made into the right carotid artery, and one of them is illustrated in figure 18a. After removal of the posterior lobe the test of similar injections was again applied, the arterial pressure being quite normal,

TABLE 5. 'SALLY'. THE EFFECT OF REMOVAL OF THE POSTERIOR LOBE OF THE PITUITARY ON THE RESPONSE TO INTRACAROTID INJECTIONS OF HYPERTONIC SOLUTIONS OF SODIUM CHLORIDE

date 1943–4	molarity and volume of injected solution		period of injection (sec.)	reduction in urine flow (c.c /min.)		response of kidney	pituitary (posterior lobe) extract equivalent of response (mU)
	M	vol. (c.c.)		from	to		
23 Nov.	0·343	20	25	nil		negative	—
1 Dec.	0·428	11	11	4·5	3·6	? small positive	—
1 Dec.	0·428	21	20	4 8	1 6	positive	about 1·0
2 Dec.	0·428	18	20	6 5	2·0	positive	> 1 0
6 Dec.			posterior lobe removed				
16 Dec.	0·428	15	15	2·8	1·6	positive	0·1
17 Dec.	0·428	21	20	3·0	1·5	positive	0·1
20 Dec.	0·428	21	20	3·5	1·5	positive	0·2
8 Jan.	0·428	21	25	4·1	3·0	positive	< 0·1
10 Jan.	0·428	21	25	2·6	1·7	positive	< 0·1

viz. 112 mm. Hg. The results are given in the lower part of the table and three of them are illustrated in figure 19 (*a*, *c* and *e*). The responses are very much diminished after the posterior lobe has been removed, and assurance is thereby given that the inhibition of water diuresis by intracarotid injection of hypertonic solutions of sodium chloride is produced by stimulation of the neurohypophysis and consequent release of antidiuretic substance into the blood stream. The expression and quantitative comparison of these responses in terms of pituitary (posterior lobe) extract are therefore justified; and when this is done, it is found that the response is diminished by some 90 % as the result of removal of the posterior lobe. The results of such assay before and after removal of the posterior lobe are given in figures 18 and 19, which show that whereas before removal of the posterior lobe about 1 mU was being released by the intra-arterial injections, after removal the quantity released by the same procedures was about 0·1 mU, the largest effect seen being equal to the response to 0·2 mU of the extract.
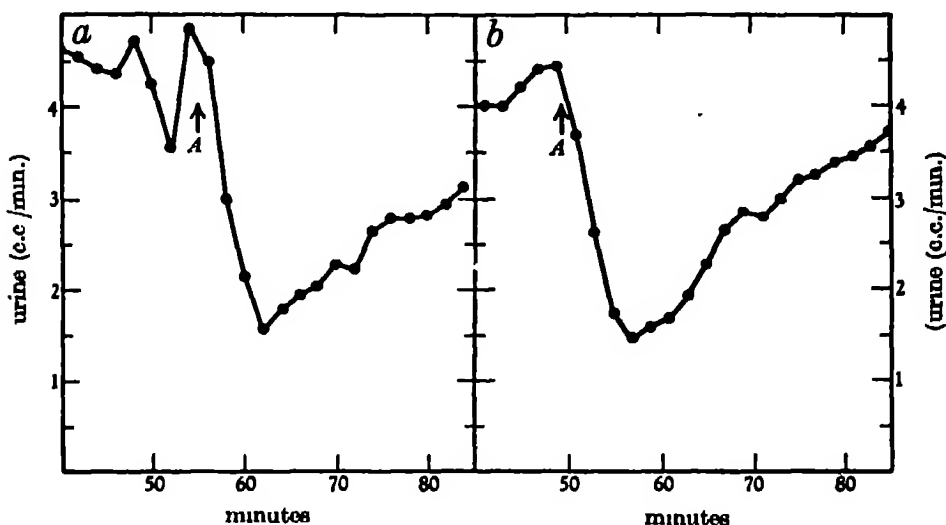
FIGURE 18. 'Sally'. *a*, 1 December 1943. At *A*, injection of 21 c.c. 0·428M-NaCl into right carotid in 20 sec. The response corresponds closely with that to 1 mU post-pituitary extract, shown in *b* where, at *A*, 1 mU in 1 c.c. 0·9 % NaCl was injected into the malleolar vein in 15 sec. Abscissae: time after the test dose of water.

(4) *The effects of short-period intracarotid injections of hypertonic solutions of sodium chloride are osmotically determined*

Comparison of the effects of hypertonic solutions of sodium chloride, dextrose and fructose. It now became important to know whether the sodium chloride was acting specifically in eliciting the responses to intracarotid injections, or by virtue of the increase which it produced in the osmotic pressure of the plasma, and to this end comparison was made of the effects of approximately isosmotic increases

produced by solutions of sodium chloride and dextrose. In figure 20 are given, on the left-hand side, three responses, one of them (*A*) being that to 10 c.c. 0·428 M-NaCl injected into the right carotid in 12 sec., the second (*B*) that to 10 c.c. 0·855 M-
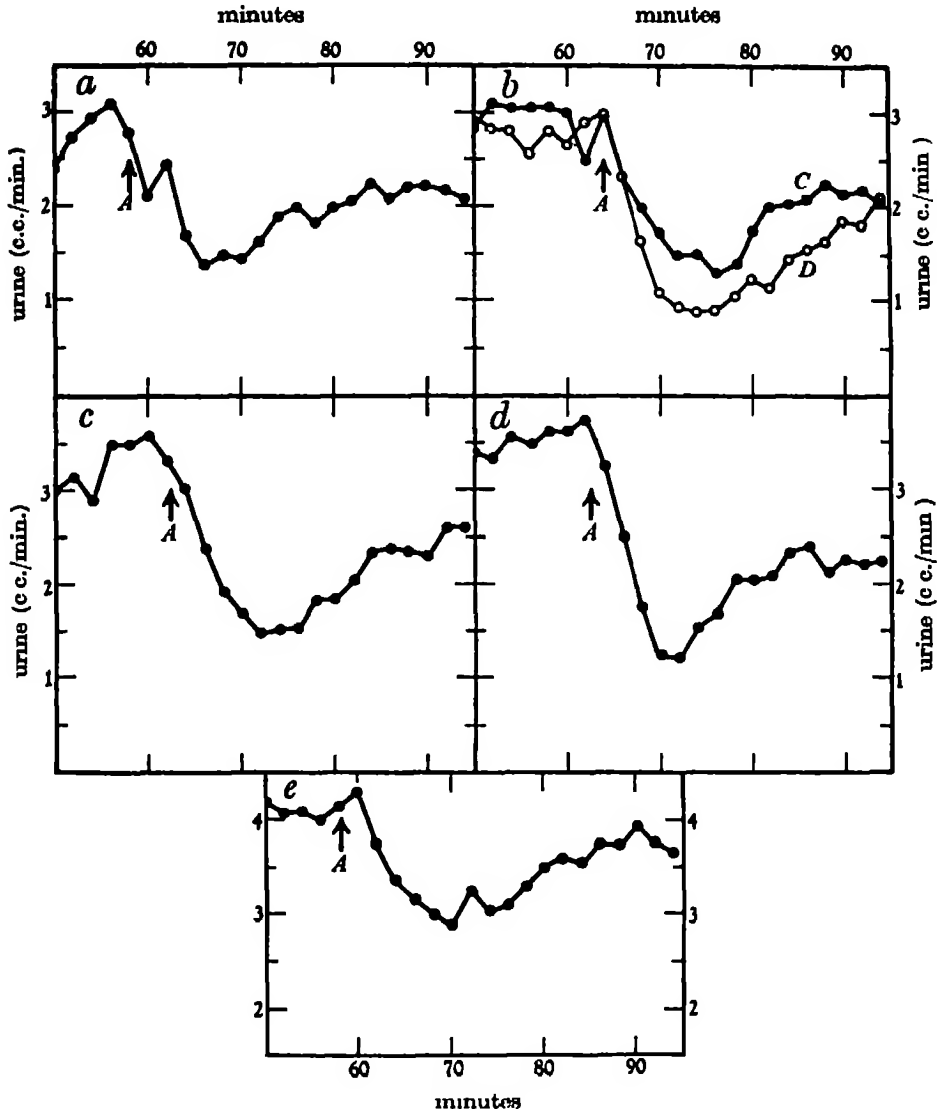
Figure 19. 'Sally'. *a*, 17 December 1943. At *A*, injection of 21 c.c. 0·428 M-NaCl into right carotid in 20 sec. The response is to be compared with those in *b* where at *A*, 0·1 mU (graph *C*) and 0·2 mU (graph *D*) in 1 c.c. 0·85 % NaCl was injected into the malleolar vein in 20 sec. *c*, 20 December 1943. At *A*, injection of 21 c.c. 0·428 M-NaCl into right carotid in 20 sec. The response is to be compared with those in *b*, and with that in *d* where at *A*, 0·4 mU was injected intravenously. *e*, 8 January 1944. At *A*, injection of 21 c.c. 0·428 M-NaCl into right carotid in 25 sec. The response is less than that to 0·1 mU. Abscissae   time after the test dose of water.

dextrose (anhydr.) injected in 11 sec., and the third (*C*) that to 2·0 c.c. 1·37 м-NaCl injected in 10 sec., the volume and strength of this last solution being such as were calculated to produce about the same increase in osmotic pressure as did the other two solutions. The three responses are very alike, and are all assayed—the assay curves (1, 2, and 3 mU) are given on the right of the figure—at 2·5 mU. The response, therefore, appears to be due not specifically to sodium chloride, but to the rise in osmotic pressure. While, however, the calculated increases in osmotic
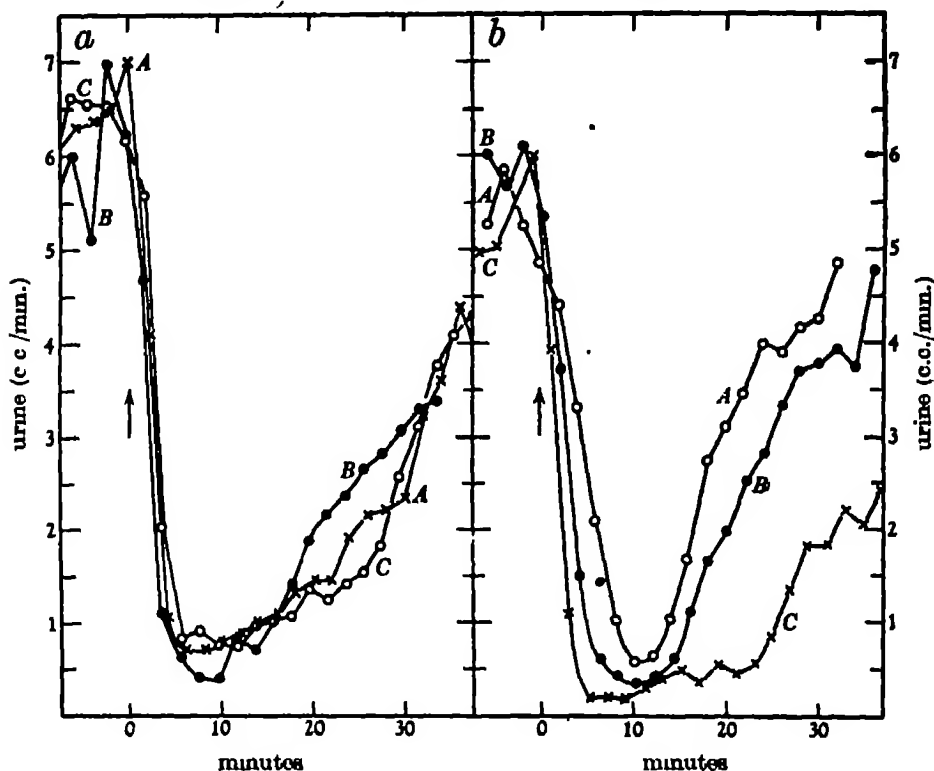
FIGURE 20. 'Nicky'. *a*, at the arrow were injected into the right carotid 10 c.c. 0·428м-NaCl in 12 sec. (graph *A*, 18 July 1944), 10 c.c. 0·855м-dextrose (anhydr.) in 11 sec. (graph *B*, 30 June 1944), and 2·0 c.c. 1·37м-NaCl in 10 sec. (graph *C*, 7 July 1944) *b*, assay curves. At the arrow were injected into the malleolar vein 1 mU (graph *A*), 2 mU (graph *B*) and 3 mU (graph *C*). Abscissae· time, the test dose of water having been given approximately 45 min. before zero.

pressure produced by the sodium chloride injections were 79 and 88 % (responses *A* and *C*), the increase produced by the dextrose injection was higher, viz. 105 %, although the response to it (*B*) was seemingly a little smaller. Nevertheless, the response to the dextrose injection was of the pituitary type, and the results suggested that the osmotic effectiveness of dextrose was less that that of sodium chloride. Moreover, a 10 sec. *intravenous* injection of dextrose was found to give a small response, a phenomenon which was not seen with either sodium chloride, sodium sulphate or sucrose, nor even with fructose, as the data in figure 21 show.

Whereas the intracarotid injection of comparable solutions of dextrose and fructose produces large and indistinguishable degrees of inhibition of urine flow, the intravenous injection produces a small inhibition in the case of dextrose alone. This small inhibition is seemingly of pituitary origin following the release of insulin, since insulin, as figure 22 shows, produces an inhibition of the pituitary type, the
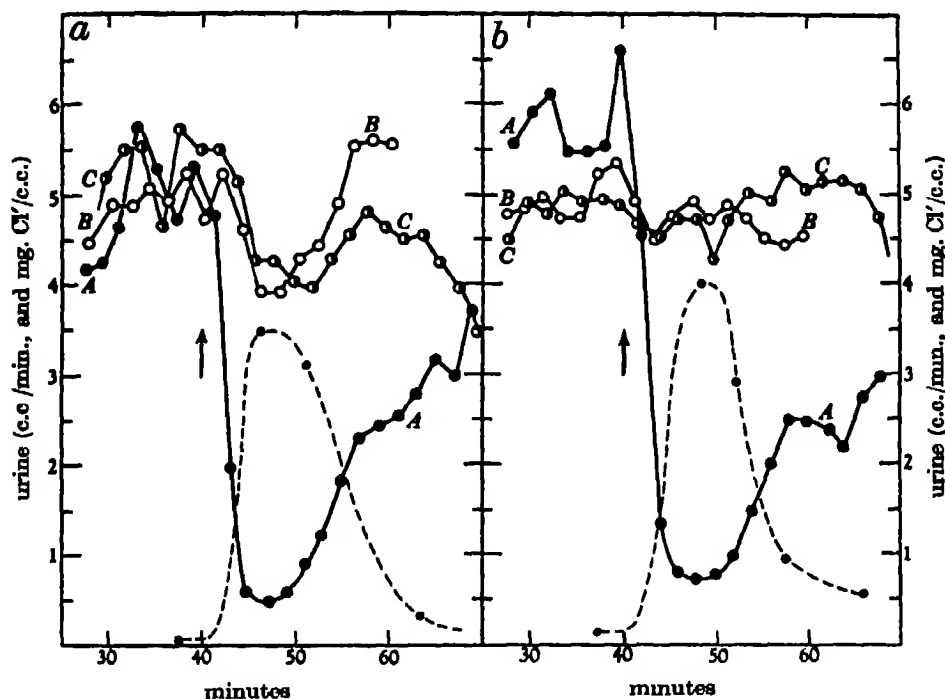
FIGURE 21. 'Nicky'. The injections were made at the arrow. *a*, 4·0 c.c. 0·916M-dextrose (anhydr.) injected in 10 sec. into the right carotid (graph *A*) and into the malleolar vein (graph *B*), and 8·0 c.c. of the same solution injected in 10 sec. into the malleolar vein (graph *C*). *b*, corresponding series of experiments with fructose. With these alone reducing sugar was briefly excreted, the urine secreted between the 2nd and 6th minute after an intravenous injection giving a small reduction when 0·25 c.c. urine was boiled with 5 c.c. Benedict's reagent. The courses of the urine chloride during the responses to the intracarotid injections are shown by the interrupted lines. The experiments were made between 3 December 1945 and 13 March 1946. Abscissae: time (approx.) after the test dose of water.

fall in urine flow being accompanied by a rise in the concentration of urinary chloride (see also figure 38). It would be remarkable, however, if insulin played an intermediate and predominantly causative role in the large response of the kidney to the *intracarotid* injection of dextrose. The magnitude of this response is not appreciably different from what would be expected on the view that the mode of action of dextrose is here an osmotic one—the 'insulin' contribution to the response, as judged by the response to the intravenous injection of dextrose, is quantitatively insignificant—; moreover, when other degrees of inhibition are produced by effecting other concentrations of dextrose in the carotid blood, the

responses are still in fair accord with those to comparable concentrations of sodium chloride (see figure 30). Confirmation or refutation of the osmotic view was, however, sought with other solutions, and the effects of these will now be described.
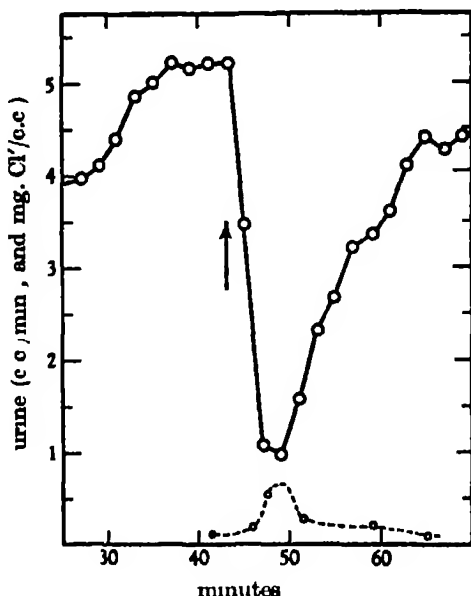
FIGURE 22. 'Nicky'. 3 February 1947. At the arrow were injected into the malleolar vein in 3 sec. 3 units of insulin in 1·5 c.c. 0·85 % NaCl. The course of the urine chloride is shown by the interrupted line. Abscissae: time after the test dose of water.

Comparison of the effects of hypertonic solutions of sodium chloride, dextrose, sodium sulphate and urea. In figure 23 (left-hand side) are shown three responses. One of them (*A*) is the response to 2·0 c.c. 1·28 M-NaCl injected into the carotid artery in 10 sec., another (*B*) that to 2·0 c.c. 0·979 M-Na₂SO₄ injected in 10 sec., and a third (*C*) that to 2·0 c.c. 2·39 M-dextrose (anhydr.), also injected in 10 sec. Comparison with the assay curves on the right of the figure (2·5 and 3·5 mU) will show that all three* responses match fairly well with that to 3·5 mU. Moreover, with all three responses the percentage of chloride in the urine rose, its course being an inverse of that of the urinary water—as with the response to post-pituitary extract—and this has been found consistently to be so. The calculated percentage increases in the osmotic pressure of the carotid blood produced by these three injections are 82, 78 and 87 respectively; and again the response to dextrose is seemingly a little less, in spite of the increase in osmotic pressure being a little greater. When, however, an approximately equal osmotic increase (84 % calc.) is produced by urea no anti-diuretic effect is seen: this is demonstrated by the graph *D*, and is a result which accords well with the high diffusibility of this substance.

* In the experiment with sodium sulphate, and in two other similar experiments, the animal gave a little sneeze just after the injection.

Comparison of the effects of hypertonic solutions of sodium chloride and sucrose.
The responses which have just been described (figure 23) were large. A more exacting
test of their osmotic determination would be furnished by a comparison of the
effects of smaller increases in osmotic pressure produced by different solutes, since
the smaller responses are more easily differentiated. This has been done with sodium
chloride and sucrose, and the results are given in figure 24. Three responses are
shown: two of them (black circles and squares) are to the intracarotid injection of
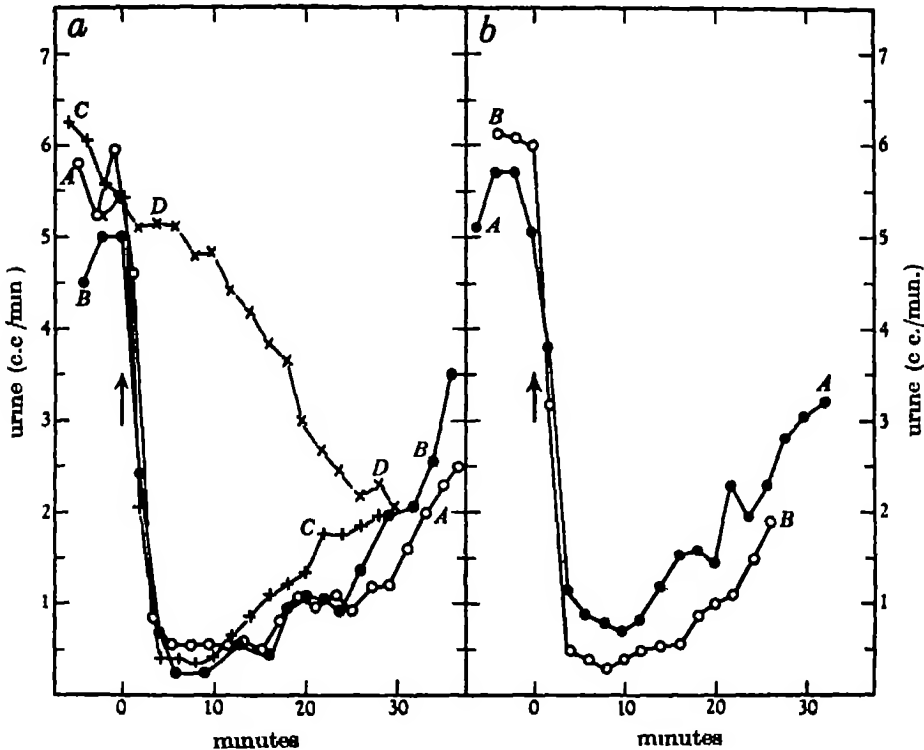
FIGURE 23. 'Nicky'. *a*, at the arrow were injected into the right carotid in 10 sec. 2·0 c.c.
1·28M-NaCl (graph *A*, 31 August 1945), 2·0 c.c. 0·98M-Na₂SO₄ (graph *B*, 30 August 1945),
2·0 c.c. 2·39M-dextrose (graph *C*, 29 August 1945), and 2·0 c.c 2·39M-urea (graph *D*, 30 August
1945). *b*, assay curves. At the arrow were injected into the malleolar vein 2·5 mU (graph *A*) and
3·5 mU (graph *B*). Abscissae· time, the test dose of water having been given approximately
45 min. before zero.

4·0 c.c. 0·514 M-NaCl in 10 sec., and the third (open circles) is to that of 4·0 c.c.
0·892 M-sucrose, also injected in 10 sec. The calculated percentage increases in the
osmotic pressure of the carotid blood during the injections are 52 and 53 respectively.
The response to sucrose lies between the two responses to sodium chloride. More-
over, the courses of the concentration of chloride in the urine during the three
inhibitory responses are identical. Seeing that the only common and equal change
in property of the carotid blood caused by the injection of these two solutions is the

5-2

increase in osmotic pressure, and that both produce quantitatively the same release of post-pituitary antidiuretic substance, the osmotic determination of the phenomenon is now beyond cavil.
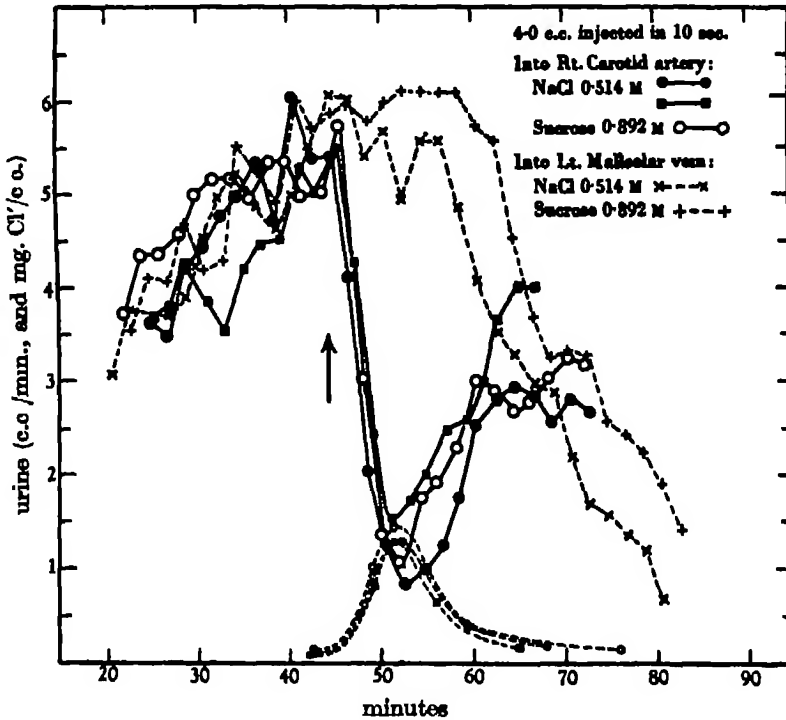
FIGURE 24. 'Nicky'. The injections were made at the arrow, on 20 December (●-●), 19 November (■-■), and 22 November 1945 (o-o). The corresponding changes in urine chloride are given by the curves at the bottom of the figure. Abscissae: time after the test dose of water.

It becomes justified, therefore, to introduce the term 'osmoreceptors' as descriptive of the autonomic receptive elements with which the neurohypophysis is functionally linked, and through whose activation the pituitary antidiuretic substance is released. The term is introduced without prejudice to any specific permeability which they may be shown to exhibit to their ionic and molecular environment; and the facts so far presented show that when they are exposed to short-lived (of the order of 10 sec.) and relatively large (50 to 100 %) increases in the osmotic pressure of their environment, their 'membrane' is relatively impermeable to sodium chloride, sodium sulphate and sucrose, less impermeable to dextrose, but freely permeable to urea.

(5) *The site of the osmoreceptors*

The results of denervating the carotid sinus and the carotid body. It was thought unlikely that the osmoreceptors would be widely distributed. A few injections of

hypertonic solutions of sodium chloride were, indeed, made into the femoral artery, but the results were indeterminate owing to sensory disturbances and the large releases of post-pituitary antidiuretic substance which accompanied them. Rather has attention been concentrated on the carotid vascular bed, on the view that the receptors would eventually be found in a precisely demarcated region within it. In the first place, they are not in the carotid sinus: the responses which we have been considering occur whether or no the sinus is denervated during the formation of the carotid loop. This will have been evident already from the results obtained with 'Sally' (figure 17), and it has been confirmed with two other animals ('Jock' and 'Whippet'). Neither are the receptors in the carotid body· this has been demonstrated on 'Alice'. Her responses to intracarotid injections of hypertonic solutions of sodium chloride in June 1943 (10·5 c.c. 0·257 M into the left carotid in 9 sec., and 11·0 c.c. 0·257 M into the right carotid in 13 sec.) have already been illustrated (figure 16). Three months later the left carotid (sinus) nerve was divided as far as possible (about 1 cm.) above the carotid sinus, the carotid body being thereby denervated. After this operation inhibitory responses were still obtained: they are illustrated in figure 25, graph *B* on the left-hand side giving the response to 11 c.c. 0·342 M-NaCl injected into the left carotid in 9 sec , and the graph on the right-hand side the response to an equal volume of the same solution injected into the right carotid in 10 sec. Denervation of the left carotid body has not affected the original similarity of the responses to like injections into the common carotid arteries. Search for the site of the osmoreceptors must be made elsewhere.
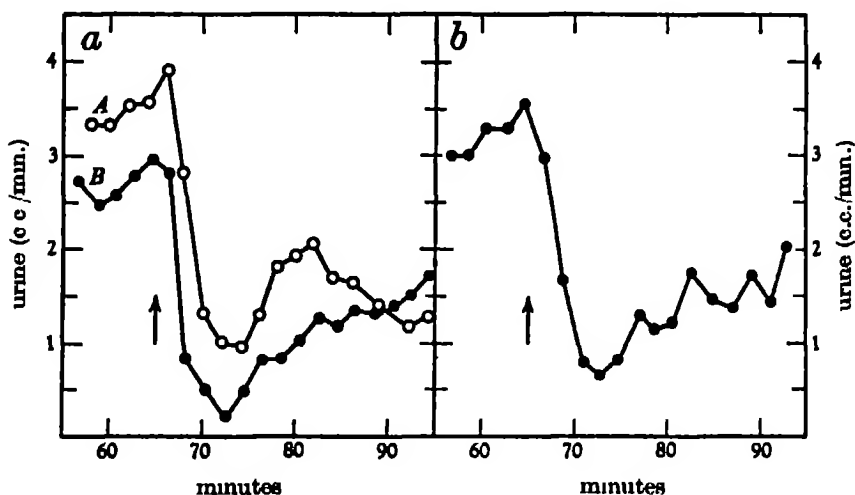
FIGURE 25. 'Alice'. The injections were made at the arrows. *a*, injections into the left carotid. 11·0 c.c. 0·257 M-NaCl in 12 sec. (graph *A*, 4 November 1943), and 11·0 c.c. 0·342 M-NaCl in 9 sec. (graph *B*, 22 November 1943). *b*, injection into right carotid. 11·0 c.c. 0·342 M-NaCl in 10 sec. (26 November 1943). Forty-four days before the first of these experiments the carotid nerve on the left side had been divided about 1 cm. above the carotid sinus. Abscissae: time (approx.) after the test dose of water.

It was decided to test the effect of ligating the internal carotid artery, and the results of this procedure will now be described.

The effects of ligating the internal carotid artery. The first attempt of this nature was made on 'Alice' 18 days after the right and left abdominal splanchnic nerves had been divided, and the right suprarenal and right abdominal sympathetic chain removed. The bifurcation of the left common carotid was exposed, and what was taken to be the internal carotid—the dissection was difficult owing to scarring from a previous operation—was divided between ligatures. When, 5 days later, the same injection as had previously been found effective was made into the left common carotid, a response not appreciably different from those obtained by a like procedure before operation was seen. One month later the lingual branch of the right glossopharyngeal nerve was divided as it curved over the caudal edge of the stylopharyngeus muscle, dissected anteriorly until it disappeared under the intermediate cornu of the hyoid, and the freed segment of nerve was removed. Seven hours after operation the animal succumbed to respiratory obstruction, and examination revealed that I had failed to tie the left internal carotid at the previous operation, two smaller vessels in the carotid angle having been ligated instead. The experiment thus became of value as a control of the effects of a successfully conducted one. This was carried out with 'Pat' (figure 13, plate 10), and the results are illustrated in figure 26. On the left-hand side of the figure are those of injections into the left, and on the right-hand side those of injections into the right common carotid. The volume of each injection was 2·0 c.c. and duration of each was 10 sec. The graphs A show the responses to 1·37 M-NaCl (left) and 1·41 M-NaCl (right) before ligation of the left internal carotid artery, the graphs B those to the same injections 6 days (left) and 7 days (right) after operation. The response to injection into the left common carotid has vanished, and that to injection into the right has become much diminished. Two days later an injection 25 % stronger (1·71 M) into the left carotid gave no response, but an injection 3 % stronger (1·45 M) into the right released more than 5 mU (the assay curves are given in figure 27). The responses obtained 23 days (left) and 24 days (right) after operation are given in the graphs C in the figure, that on the left being the response to 1·71 M-NaCl, that on the right the response to 1·43 M-NaCl. While a 1·5% increase in the strength of the solution injected into the right common carotid is now causing a response which is indistinguishable from that obtained before operation, a 25 % increase in that injected into the left is producing a response which is very much smaller, and one, moreover, which is in all probability of emotional origin seeing that intense lip-smacking followed the injection. The calculated percentage increases in the osmotic pressure of the common carotid blood which elicited the responses A before operation are 106 (right) and 103 (left), and those which elicited the responses C after operation are 108 (right) and 132 (left). It is reasonable to suppose that the osmoreceptors are bilaterally placed; and these experiments show that ligation of the left internal carotid has virtually protected the ipsilateral group from the influence of very large increases in the osmotic pressure of the blood in the common carotid artery.

The receptors lie, therefore, in the vascular bed normally supplied by the internal carotid artery. Moreover, it was observed that the phenomenon of 'lip-smacking', resulting presumably from the increased sodium chloride content of the blood supplying the taste-buds, was much intensified when, after ligation of the left internal carotid, the strong (1·71 M) solutions of sodium chloride were injected into the left common carotid. Were the osmoreceptors supplied by the external carotid
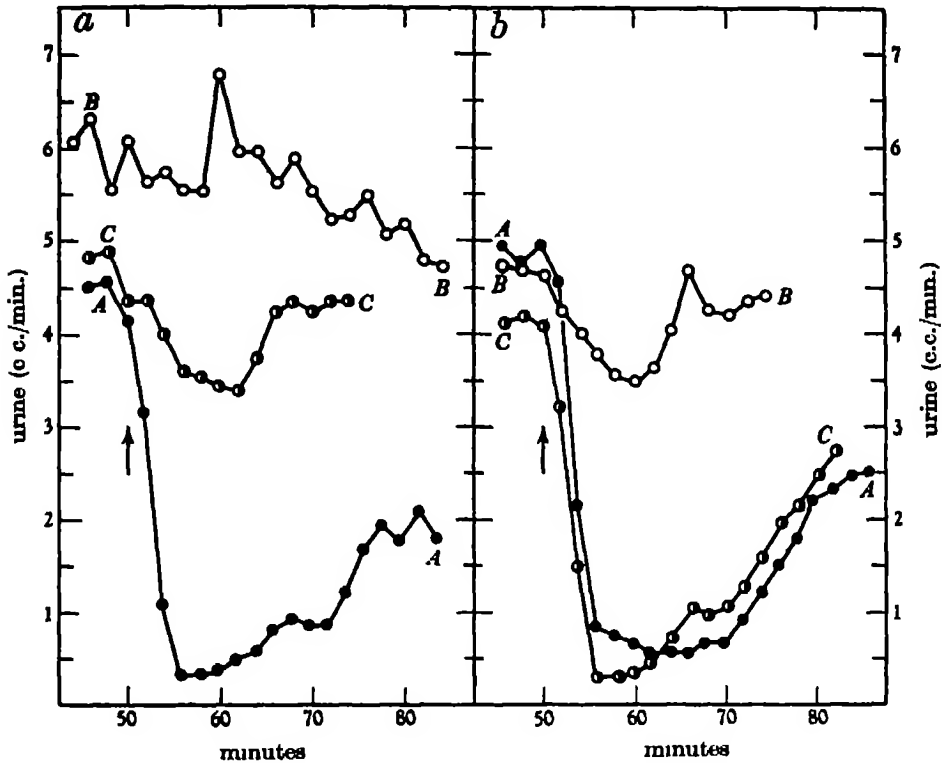
FIGURE 26. 'Pat'. The injections were made at the arrows, the volume and period of each being 2·0 c.c. and 10 sec. *a*, injections into the left common carotid, 1·37 M-NaCl (graph *A*) before ligation of left internal carotid; 1·37 M-NaCl (graph *B*) 6 days after operation, 1·71 M-NaCl (graph *C*) 23 days after operation. *b*, injections into the right common carotid; 1·41 M-NaCl (graph *A*) before ligation of left internal carotid; 1·41 M-NaCl (graph *B*) 7 days after operation; 1·43 M-NaCl (graph *C*) 24 days after operation. Abscissae: time (approx.) after the test dose of water.

artery, a big release of post-pituitary antidiuretic substance would be expected to have sprung from these injections: we have seen that this was not so. It remains to consider the cause of the much smaller decrease in response to injections into the right common carotid artery when the left internal carotid had been tied. The result of this operation will be to produce in the circle of Willis a putative fall in pressure which is immediately compensated by an increased inflow through the basilar and right internal carotid arteries. The readiness and efficiency of this

compensation are evident from the facts, already alluded to, that occlusion of both common carotids in the dog causes no change in the appearance of the retinal vessels, and that when a solution of Evans's blue is injected into the common carotid of one side, while on the other side the fundus oculi is being observed and the common carotid occluded, the dye is seen to pass through the retinal vessels. It is to be expected, then, that ligation of one internal carotid artery will be accompanied by an increased blood flow through the contralateral internal and common carotids; and this would seem to be a reasonable explanation for the decreased response to injections into the right common carotid after the left internal had been tied.
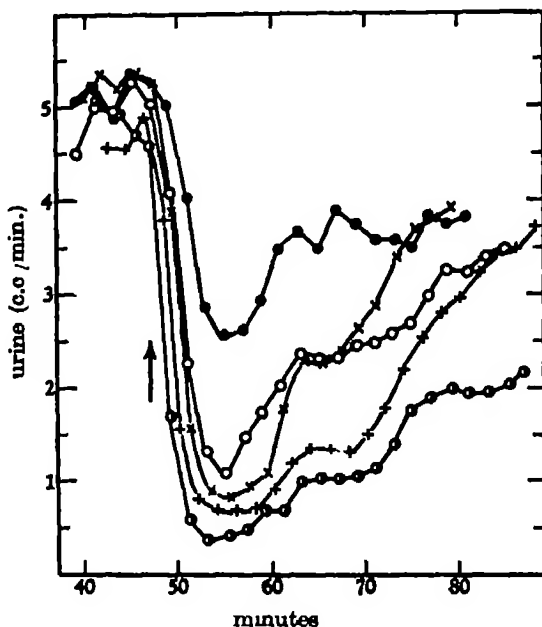
FIGURE 27. 'Pat'. Assay curves. The injections were made into the malleolar vein at the arrow, and the duration of each was 10 sec. 0·5 mU ●-●; 1·0 mU o-o; 1·5 mU ×-×; 2·0 mU +-+; 5·0 mU ◑-◑. Abscissae: time (approx.) after the test dose of water.

There is evidence that the effects described above were diminishing. It may be that the gradual widening of the short arterial anastomoses which, in confirmation of the work of Bouckaert & Heymans (1935), I have observed between the maxillary artery, as it runs along the lateral border of the maxillary division of the fifth cranial nerve, and the ophthalmic branch of the internal carotid, was responsible for this. In this connexion the results of the following perfusion experiment are instructive. In an animal which had recently died, I tied the left internal carotid just above the bifurcation of the common carotid; and after exposing the right internal carotid at the base of the brain, I tied it inside the dura and just before it gave off the posterior communicating artery, i.e. distal to its large ophthalmic branch. The arch of the aorta was then opened, a cannula tied into the superior

vena cava, and the common carotid arteries were connected with the apparatus, already described, for infusion of gelatin masses into the head and neck circulation. After the tissues had been perfused with saline, formaldehyde-saline, and saline again, carmine-gelatin was infused into the left common carotid and prussian blue-gelatin into the right. On dissection there was revealed a sharp mid-sagittal line of demarcation between the carmine-containing tissues on the left and the prussian blue-containing tissues on the right. The whole of the brain, however, including the circle of Willis, contained the carmine mass only. Although, on the right side, the blue mass filled the extradural part of the internal carotid, it was prevented by the ligature from entering the circle of Willis; whereas, on the left side, the carmine mass had passed via the external carotid, and the anastomosing short vessels, described above, to the ophthalmic branch of the internal carotid, and so, by reversal of the normal direction of flow in this branch, had reached the internal carotid and the circle of Willis. If, then, one internal carotid were tied intradurally in the living animal, one would expect the responses to a raised osmotic pressure in the common carotid blood of the same side to be permanently suppressed. Such a procedure is, I believe, a surgical possibility in the dog.

One other observation merits record in connexion with the effect of ligation of the internal carotid artery. We have seen that, with 'Sally', removal of the posterior lobe was followed by a big diminution in the response to intracarotid injections of hypertonic solutions of sodium chloride: the small residual inhibitions have been illustrated in figure 19. Two days after the last of these small responses had been obtained, the right internal carotid was divided between ligatures just beyond its origin. Seven, nine and ten days after this, the same injections as were producing, before operation, small inhibitions of urine flow, failed to cause any inhibition whatsoever. 'Lip-smacking' towards the end of the injection period was, however, just as marked as in the experiments before ligation of the internal carotid. These results confirm those obtained from 'Pat'.

It is to me an unattractive hypothesis to suppose that the endings of the supra-optico-hypophysial tract themselves exhibit osmoreceptive properties, though it may become necessary to test this by measuring the antidiuretic response to an intracarotid injection before, and as soon as possible after high section of the tract, that is to say, before degeneration has spread to the nerve endings. Meantime it would seem justifiable to search for structures linked with the supraoptic cytons, and having such histological characters as would reasonably endow them with the properties which experiment has already revealed. Although a preliminary and cursory examination of the supraoptic nucleus in the dog has under some circumstances disclosed roughly spherical vesicles among the cytons, further reference to them is best relegated to discussion, since there is at present no evidence that they are in any way concerned in the nexus of events initiated by a rise in the osmotic pressure of the blood in the internal carotid artery and culminating in the release of post-pituitary antidiuretic substance. To the relationship between these two factors it now becomes convenient to turn.

(6) The effects of varying increases in the osmotic pressure of the carotid blood on the release of post-pituitary antidiuretic substance

The first experiments in this connexion were made on the dog 'Sally' at a time when she had been subjected to no surgical procedures other than perineotomy and the formation of two carotid loops. Details of the intracarotid injections are given in table 6, and the responses to five of these have already been illustrated in figure 17. The responses were all carefully assayed—the assay curves are shown in figure 28—and their post-pituitary extract equivalents have been plotted against the percentage increases in the osmotic pressure of the carotid blood. The result of this is given in figure 29. The figure suggests that there is a fairly simple relationship between osmotic pressure increment and amount of post-pituitary antidiuretic substance released, and that—excepting the two observations at 35 and 36 % increments—the period of exposure of the osmoreceptors to a constant increase in osmotic pressure is strongly operative. The asterisked observation is interesting in that the injection, the results of which it represents, was made while the contralateral carotid was being occluded the amount of antidiuretic substance released was less than that released by a closely similar injection when the contralateral carotid was free, a result which would be expected from the increased carotid blood flow in the former instance (see table 1, p 50, table 3, p. 53; table 4, p. 54).

TABLE 6. 'SALLY'. THE POST-PITUITARY EXTRACT EQUIVALENTS OF RESPONSES TO INTRACAROTID INJECTIONS OF SOLUTIONS OF SODIUM CHLORIDE, AND THE PERCENTAGE INCREASES (CALC.) IN THE OSMOTIC PRESSURE OF THE CAROTID BLOOD

date 1943	carotid injected	strength (M) and vol. (c.c.) of solution		period of injection (sec.)	post-pituitary extract equivalent of response (mU)		increase (calc.) in osmotic pressure of carotid blood (%)
		(M)	(c.c.)		by assay	by closer judgment	
13 Aug.	left	0 257	10	12	nil	nil	25·3
19 June	left	0·257	10	10	0·1	0·1	29·6
10 May	left	0·342	12	60	0·4	0 4	11·2
17 Aug.	left	0·257	20	16	0·5–1	0·6	36·1
27 May	left	0·257	11	12	1	0·8	35·1
19 May	right	0·342	12	15	1–2	1·5	42·6
5 Sept.	left	0 342	20	25	2	2·0	42·6
26 May	left	0·342	11	9	2	2·0	63·5*
20 Sept.	right	0·342	20	21	> 2	2 5	50 5
14 May	left	0·342	12	10	> 2	2·5	62·5

* This injection was given while the right carotid was being occluded.

The results obtained with 'Sally' made it seem worth while to pursue the investigation further, with a view first to eliminating the factor of variation in the period of exposure of the osmoreceptors to a raised osmotic pressure, and secondly to
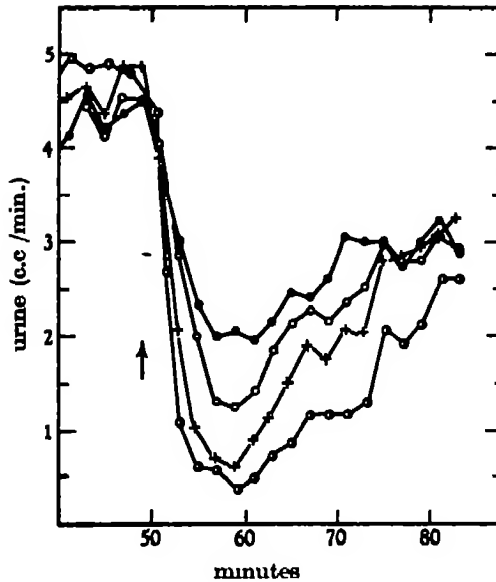
FIGURE 28. 'Sally'. Assay curves. The injections were made into the malleolar vein at the arrow. 0·5 mU ●-●; 1·0 mU o-o; 2·0 mU +-+; 5·0 mU ◑-◑. Abscissae. time (approx.) after the test dose of water.
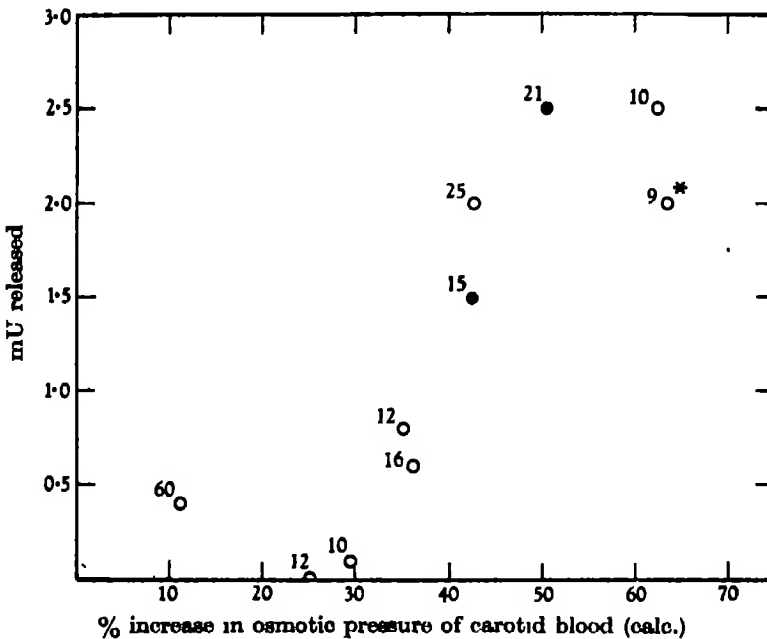
FIGURE 29. 'Sally'. The results of ten intracarotid injections of solutions of sodium chloride. Open circles, injections into the left carotid; black circles, injections into the right. The number against each circle is the period (sec.) of the injection. *, during this injection the right common carotid was occluded.

lessening the possibility of the effects of the intracarotid injections being influenced by changes in carotid blood-flow accompanying incidental changes in sympathetic activity. The next series of observations, therefore, was made with a sympathectomized animal, and the period of the intracarotid injections was kept as constant as possible.

The relation between increase in the osmotic pressure of the carotid blood and release of post-pituitary antidiuretic substance. The observations were made on 'Nicky' over a period of seven months, and details of the injections and of their effects are given in table 7. All the inhibitory responses were assayed before the increases in osmotic pressure were calculated. The periods of the injections lay between 9 and 12 sec. in twenty-seven of the thirty-two observations, and the results of these have been plotted in figure 30. The relationship between increase in osmotic pressure and amount of antidiuretic substance released when injections are made into the right carotid, runs a course different from that when injections are made into the left. When the osmotic pressure is raised beyond a certain threshold, manifestations of
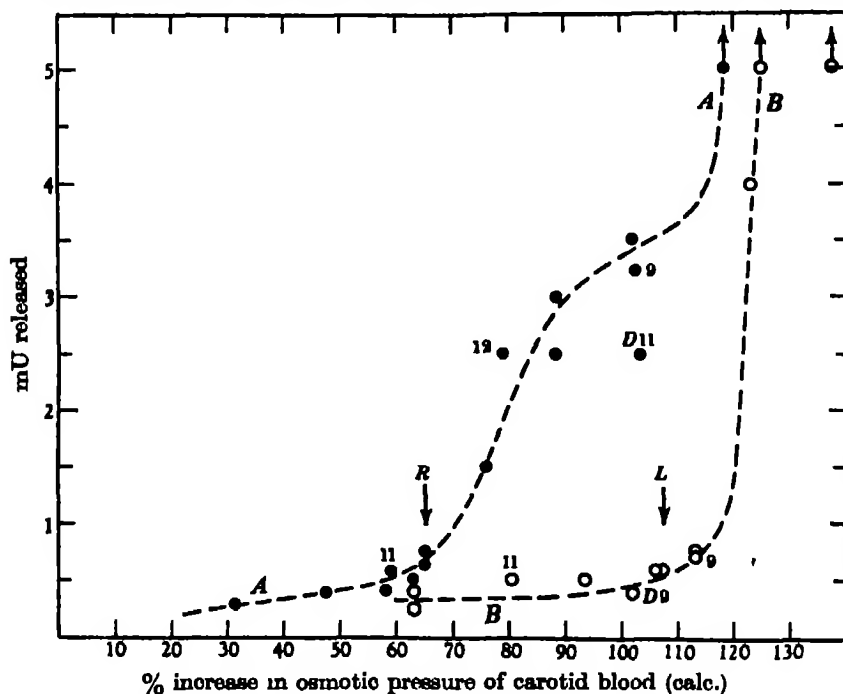
FIGURE 30. 'Nicky'. The relation between increase in osmotic pressure of the carotid blood and amount of post-pituitary antidiuretic substance released. The period of injection was mostly 10 sec., and only observations in which the period lay between 9 and 12 sec. have been included; where the period was other than 10 sec., this is indicated by the figures beside the circles. D = dextrose solution, the other solutions being sodium chloride. The black circles give the results of injections into the right carotid, the open circles those of injections into the left. The arrows R and L mark the onset of 'lip-smacking' following injections into the right and the left carotid. All the observations were made within the 7 months' period June 1944 to January 1945.

TABLE 7. 'NICKY'. THE FIGURES ABOVE THE SPACE RELATE TO INJECTIONS INTO THE RIGHT CAROTID ARTERY, THOSE BELOW THE SPACE TO INJECTIONS INTO THE LEFT

The data in the fifth and sixth columns for injection periods between 9 and 12 sec. are plotted in figure 30

date	strength (M) and vol. (c.c) of injected solution		period of injection (sec.)	post-pituitary extract equivalent of response (mU)	percentage increase (calc.) in osmotic pressure of carotid blood	remarks on associated manifestations
	(M)	(c.c.)				
5 Jan. 1945	NaCl 0·565	2·0	8·5	0·3	31·4	no lip-smacking
27 July 1944	NaCl 0·342	10·0	17	0·2	39·7	no record
3 Jan. 1945	NaCl 0·804	2·0	10	0·4	47·3	no lip-smacking
11 Sept. 1944	NaCl 0·941	2·0	10	0·4	58·1	no lip-smacking
29 July 1944	NaCl 0·342	10·0	11	0·6	59·8	no record
11 Aug. 1944	NaCl 1·027	2·0	10	0·5	63·2	no lip-smacking
1 Aug. 1944	NaCl 0·342	10·0	10	0·75	65·0	a little lip-smacking
5 Aug. 1944	NaCl 0·942	10·0	10	0·85	65·0	no lip-smacking
25 Sept. 1944	NaCl 1·198	2·0	10	1·5	75·8	a little lip-smacking
18 July 1944	NaCl 0·428	10·0	12	2·5	79·1	a little lip-smacking
7 July 1944	NaCl 1·369	2·0	10	2·5	88·1	a little lip-smacking
29 Sept. 1944	NaCl 1·369	2·0	10	3·0	88·1	definite lip-smacking
12 June 1944	NaCl 0·428	5·0	5	0·5	93·5	no record
5 July 1944	NaCl 1·711	1·8	10	3·5	101·8	definite lip-smacking
16 June 1944	NaCl 0·428	10·0	9	3·25	102·9	lip-smacking and transitory movement at end of injection
30 June 1944	dextrose 0·855	10·0	11	2·5	104·7	no record
5 Jan. 1945	NaCl 1·711	2·0	9·5	5	118·4	lip-smacking and struggle
28 Sept. 1944	NaCl 1·027	2·0	10	0·4	63·2	no lip-smacking
3 Jan. 1945	NaCl 1·027	2·0	10	0·25	63·2	no record
7 July 1944	NaCl 1·369	2·0	11	0·5	80·1	no record
17 June 1944	NaCl 0·428	10·0	10	0·5	93·5	no lip-smacking
29 July 1944	NaCl 0·513	4·0	5	0·3	99·6	no record
23 June 1944	dextrose 0·855	8·0	9	0·4	102·2	no record
12 June 1944	NaCl 0·428	5·0	4·5	0·4	102·9	no record
16 June 1944	NaCl 0·428	20·0	18	1·0	102·9	lip-smacking and head-movement
10 Aug. 1944	NaCl 0·492	10·0	11	0·8	105·8	no lip-smacking
3 Aug. 1944	NaCl 0·471	10·0	10	0·6	107·2	a little lip-smacking
4 July 1944	NaCl 1·711	1·8	9	0·75	112·8	a little lip-smacking
28 Sept. 1944	NaCl 1·711	2·0	10	0·75	112·8	a little lip-smacking
1 Aug. 1944	NaCl 0·513	10·0	10	4	122·8	lip-smacking and struggle for 10 sec. after injection
15 Aug. 1944	NaCl 1·882	2·0	10	>5	125·3	momentary severe struggle after injection
8 July 1944	NaCl 2·053	2·0	10	>5	137·5	momentary severe struggle after injection

central nervous stimulation appear in the form of movement of the head, stiffening of the face and neck musculature or a more generalized muscular response, signs which I imagine to result from an action of the raised osmotic pressure on cortical areas supplied by the middle cerebral artery. Such manifestations are of course associated with the release of large amounts of pituitary antidiuretic substance: with this release we are now not concerned. As we trace in the figure the effects of graded increases in the osmotic pressure of the blood in the right carotid artery, we see that the osmotic release of antidiuretic substance increases up to a maximum of some 3·5 mU before signs of cortical stimulation appear. With injections into the left carotid artery, on the other hand, these signs appear when a maximum osmotic release of some 0·75 mU is exceeded. There is at present no factual basis for an interpretation of this difference.

The hypothesis that, of the total number of osmoreceptors, the right internal carotid is supplying a proportion much greater than is the left, is in probable conflict with the observation that the onset of 'lip-smacking' coincides with the release of roughly the same amount of post-pituitary antidiuretic substance into whichever common carotid the injections are made. Moreover, the left retinal vessels were not more quickly reached by Evans's blue injected into the right carotid while the left was occluded, than were the right retinal vessels when the injection was made into the left carotid while the right was occluded (see table 4, p. 54).

The alternative view, that the observed difference between the effects on the neurohypophysis of graded increases in the osmotic pressure of the blood in the right and in the left common carotid results from an underestimate of the volume flow through the left carotid—and this is supported by the fact that the diameter of the left carotid in this animal is palpably a little larger than that of the right—requires the movement of the curve B to the left and, if we accept the onset of lip-smacking as indicative of the same increase in the osmotic pressure of the blood of both common carotid arteries, to such an extent that the arrow L becomes super-imposed on the arrow R. In this event manifestations of cortical stimulation arise from a much smaller increase in the osmotic pressure of the blood in the left than in the right internal carotid. Possibly some aid in the interpretation of these observations will be furnished at a later stage by examination of the Circle of Willis and of the mode of branching and the distribution of the two internal carotid arteries; but for the moment, and in the absence of additional data, it is futile to carry speculation further.

With injections into the right carotid artery (graph A, figure 30) those responses which are solely of osmotic origin follow a course which appears to be sigmoid. While such a relation is commonly found between concentration of drug and incidence of response in a population of cells or individuals, it is not infrequently encountered between concentration of drug and degree of action in a tissue capable of a graded response, e.g. between the concentration of potassium chloride and the percentage inhibition of the isochoric response of the frog's isolated heart (Clark

1933), and between the concentration of acetylcholine and the percentage inhibition of the isometric response of the frog's ventricle (Clark 1926). The sigmoid character of the response of the neurohypophysis to graded increases in the osmotic pressure of the carotid blood is disclosed by increases which are far greater than those which could operate as physiological determinants. The osmotic origin of such responses, however, gives rational purpose not only to the search for receptors with befitting histological features, but also to the investigation of the properties of these receptors as revealed by exposure to smaller and more prolonged increases in the osmotic pressure of their environment, and finally to an assessment of their role in the 'co-ordinated maintenance' (Haldane 1931) of the living animal. To this investigation and assessment I now propose to turn.

(7) *The effects of 10-minute exposure of the osmoreceptors to increases in the osmotic pressure of the carotid blood*

A fair extrapolation of the graph A (figure 30) to the left meets the X and Y axes at about zero; and if the osmoreceptors fail to accommodate to a protracted increase in the osmotic pressure of their environment, a 5 % increase in this pressure operating over a period of 10 min. will, provided the rate of destruction is not excessive, liberate post-pituitary antidiuretic substance in an amount which is readily detectable. That the receptors do not accommodate during short-period exposure (5 to 20 sec.) was suggested by the observations on 'Sally' (figure 29) and supported by those on 'Nicky' (table 7); and observations made in this connexion on the latter animal earlier in her career than those recorded in table 7 are illustrated in figure 31. On the left are shown two responses to the intracarotid injection of 0·428 M-NaCl. A is the response to 5·5 c.c. injected in 4·5 sec., and B that to 11·0 c.c. injected in 9 sec. The assay curves (0·25, 0·5, 1·0 and 2·0 mU) are shown on the right. Comparison of the responses on the left with those on the right will show that the 4·5 sec. exposure is associated with the release of 0·5 mU, and the 9 sec. exposure with the release of 1·5 mU. Doubling the period of exposure of the osmoreceptors to the same increase in sodium chloride concentration causes, therefore, the release of at least double the quantity of post-pituitary antidiuretic substance.

Before the investigation of the effects on the neurohypophysis of infusing hypertonic solutions of sodium chloride into a carotid artery for periods of the order of 10 min. was proceeded with, it was necessary to determine the course of the aortic blood chloride during the infusion of such solution into the carotid artery on the one hand and into the malleolar vein on the other. Chloride determinations on samples of blood from the contralateral carotid taken at intervals during the 10 min. period of infusion (these samples being representative of the aortic blood), showed that the aortic blood chloride followed the same course irrespective of whether the infusion were made into the carotid artery or into the malleolar vein. That this is so is shown by the results given in figure 32. In so far, therefore, as the effects of an increase in the aortic blood chloride are concerned, intravenous

infusions may be used as controls of intracarotid infusions; and I shall assume that
the same holds with infusion of other solutes.

Now it was found that when a concentrated infusion of sodium chloride was
made into the carotid over a period of some 7 min., the inhibition of urine flow
occurred earlier than when the same infusion was made into the malleolar vein; and
that as the strength of the infused solution was reduced this difference became
greater. These facts are illustrated in figures 33 and 34. In the experiments of
figure 33, sodium chloride was infused into the right carotid artery (black circles)
for 6 min. 48 sec. at a rate of 3·0 mg./sec., and into the malleolar vein (open circles)
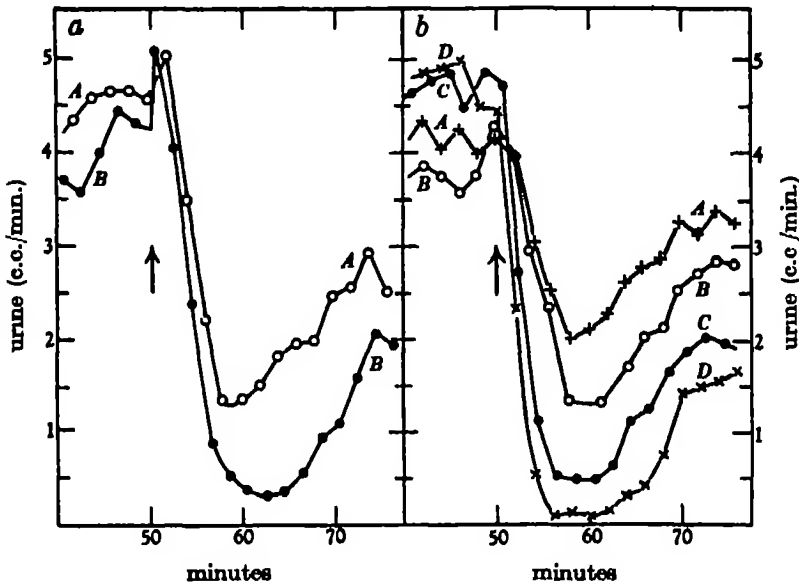
FIGURE 31. 'Nicky'. To illustrate the effect of the period of intracarotid injection of a hyper-
tonic solution of sodium chloride on the response of the neurohypophysis during water diuresis.
a, at the arrow were injected into the right carotid 5 5 c.c. 0·428M·NaCl in 4 5 sec. (graph A),
and 11 c.c. of the same solution in 9 sec. (graph B). b, at the arrow were injected into the
malleolar vein 0·25 mU (graph A), 0·5 mU (graph B), 1·0 mU (graph C) and 2·0 mU (graph D).
The experiments were made between 8 and 16 March 1944. Abscissae: time (approx.) after
the test dose of water.

for 7 min. 39 sec. at the same rate. In the former instance the urine flow begins to
fall precipitously some 3 min. after the infusion has started, whereas in the latter
instance the fall, slightly less precipitous, begins some 4 min. later. When, as in
figure 34, the rate of infusion of sodium chloride is reduced to about one-third of that
employed in the experiments of figure 33, the difference between the effect of the
intracarotid and that of the intravenous infusion becomes more evident. In both
instances the cause of the earlier onset of the inhibition of urine flow when the
infusion is made into the carotid artery, must be attributed to the increase in osmotic
pressure of the blood in the vascular bed supplied by the carotid over and above

that of the aortic blood. Such increase in the experiment of figure 33 is calculated to be 18·0 %, and in the experiment of figure 34, 5·45 %. When the response to this latter increase in osmotic pressure is assayed by incorporating in the intravenous infusion an appropriate quantity of post-pituitary extract, it is found to be a little smaller, as figure 34 shows, than that corresponding to a release of pituitary anti-diuretic substance at a rate of 1·67 μU/sec. The urine chloride during these two
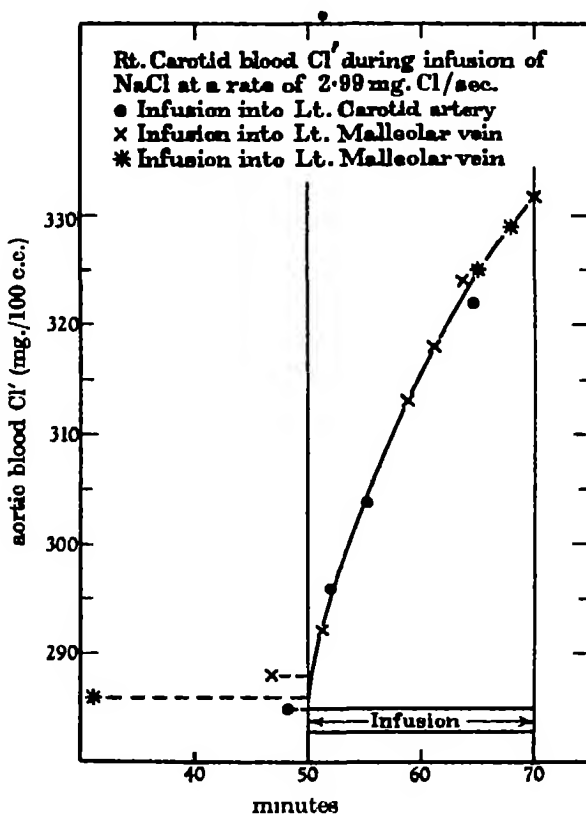
FIGURE 32. 'Nicky'. To illustrate the course of the aortic blood chloride during intracarotid and intravenous infusion of 4·705 M-NaCl. The solution was infused at the rate of 1·08 c.c./min. The experiments were made between 15 March and 30 April 1945. Ordinates· right carotid (aortic) blood-chloride in mg. Cl'/100 c.c. Abscissae: time (approx.) after the test dose of water.

responses rose from 0·2 to a maximum of 3 mg. Cl'/c.c., and the time courses of the urine chloride were similar. Six months later a repetition of this experiment gave a response whose equivalent was a little more than 1·67 μU/sec., and it was then decided to compare this response with the effects of infusing osmotically comparable solutions of dextrose.

Comparison of the effects of osmotically comparable infusions of sodium chloride and dextrose. All the arterial infusions were made into the right carotid. As was

expected from the results of the short-period injections, two striking differences appeared between the effects of infusions of sodium chloride and dextrose: the intravenous infusion of dextrose produced a definite inhibition of urine flow, and the intracarotid infusion an inhibition which was smaller than that produced by a comparable infusion of sodium chloride. In figure 35 the graph A on the left-hand side represents the response to an infusion of 1·655M-dextrose at 1·09 c.c./min. into
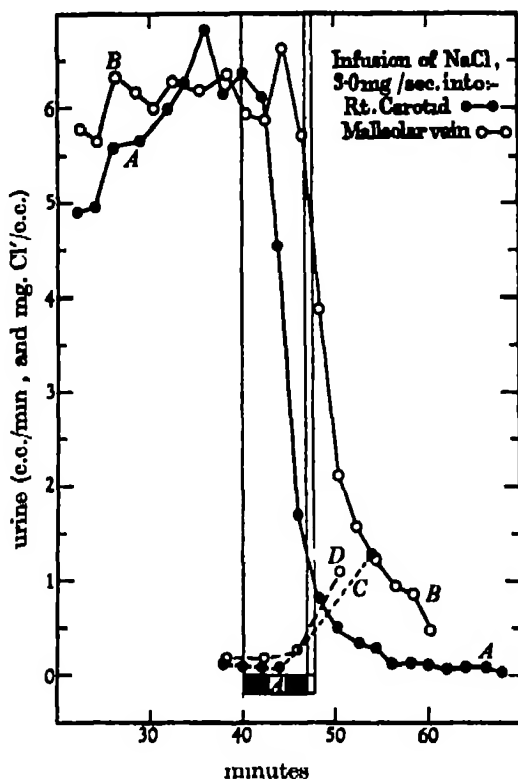
FIGURE 33. 'Nicky'. To illustrate the earlier onset of the inhibition of urine flow when a concentrated solution of sodium chloride is infused into a carotid artery than when infused into the malleolar vein. The rectangles A at the bottom of the figure are the respective periods of infusion. Graph A, the response to intracarotid infusion, graph B that to intravenous infusion. The corresponding changes in urine chloride are given by the graphs C and D The solution infused was 2·823 M-NaCl at 1·08 c.c./min. The experiments were made on 14 and 15 May 1945. Abscissae: time (approx.) after the test dose of water.

the right carotid artery, and on the right-hand side the response to the same infusion into the malleolar vein: the latter response, though definite, is smaller than the former. The graphs B in the figure illustrate the same facts, but here a stronger solution—1·860M infused at the same rate as before—was used: the calculated local increase in the osmotic pressure of the blood during the intracarotid infusion was 5·78 %. The response to this infusion, as comparison with graph C will show, is a little less than that to the intravenous infusion of the weaker

(1·665 M) solution of dextrose along with post-pituitary* extract 1·67 μU/sec. With sodium chloride, however, this release was being effected by an increase in osmotic pressure of 5·45 %. This comparison between the effects of sodium chloride and dextrose was again made some 6 months later when the animals' sensitivity to intracarotid infusions of sodium chloride was greater. The results are shown in figures 36 and 37. On the left-hand side of figure 36 are given two responses to an
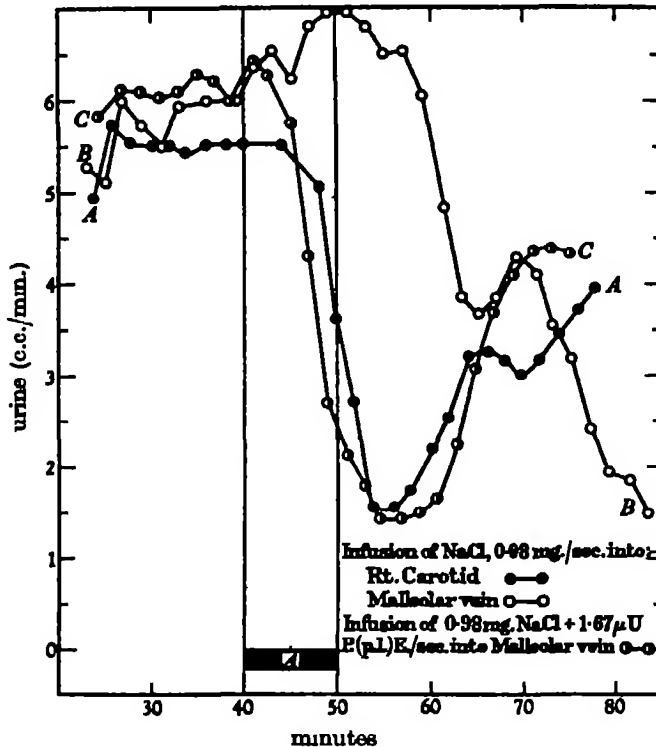
FIGURE 34. 'Nicky'. To illustrate the comparative effects of a weaker intracarotid and intravenous infusion than that employed in the experiments of figure 33, and the assay of the response to the intracarotid infusion. The rectangle A covers the period of the infusions. Graph A, the response to intracarotid infusion, graph B that to intravenous infusion, and graph C that to intravenous infusion + post-pituitary extract 1 67 μU/sec. The solution infused was 0·933 M-NaCl at 1·08 c.c./min. The experiments were made in May 1945. Abscissae: time (approx.) after the test dose of water.

intracarotid infusion of sodium chloride such that the local increase in the osmotic pressure of the blood was 5·05 %. Comparison with the assay curves on the right-hand side (1·67 and 3·33 μU/sec.) will show that the intracarotid infusions were causing the release of post-pituitary antidiuretic substance at a rate which is much

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* When a solution of 2·5 mU/c.c. 2·5 M-dextrose was stood at room temperature for 90 min., the response to 1 c.c. injected intravenously in 10 sec. was indistinguishable from that to 2·5 mU in 1 c.c. 0·85 % NaCl.

closer to 3·33 than to 1·67 μU/sec. In figure 37 are given the results of a series of strictly comparable experiments with dextrose: the two responses on the left-hand side are to an intracarotid infusion such that the local increase in the osmotic pressure of the blood was again 5·05 %, and the responses in the graphs on the right are to the same infusion but given intravenously without (A and B) and with (C) post-pituitary extract 1·67 μU/sec. This last is a little greater than that to the larger of the two responses to intracarotid infusion. The results given in figures 36
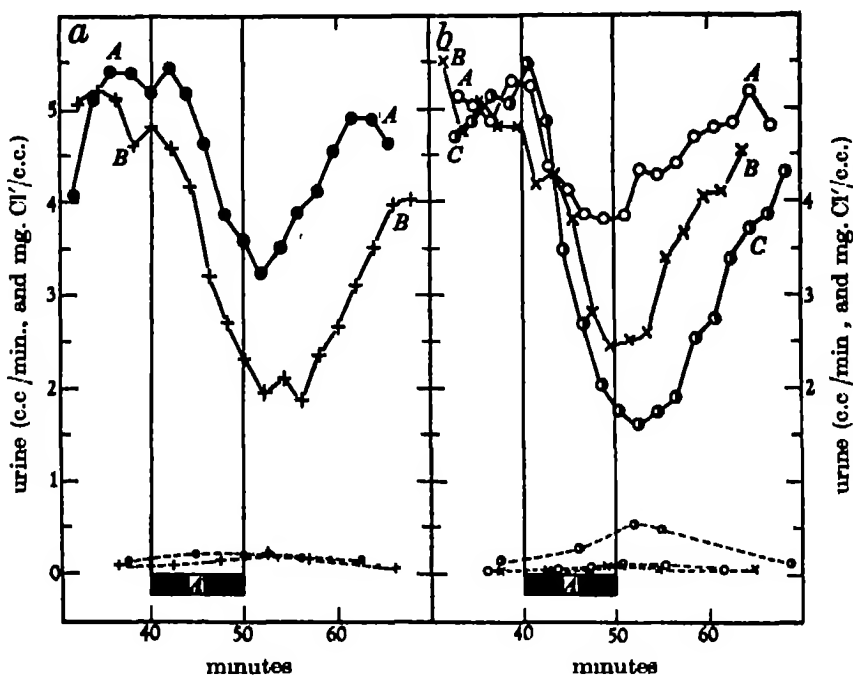
FIGURE 35. 'Nicky'. To illustrate the effects of intracarotid and intravenous infusions of dextrose. a, responses to intracarotid infusions. b, responses to intravenous infusions. The rectangles A cover the period of the infusions. Graphs A and B give the responses to infusions, at 1·09 c.c./min., of dextrose 1·665 and 1·860M respectively. Graph C gives the response to an infusion of dextrose 1·665M + post-pituitary extract 1·67 μU/sec. The courses of the concentration of chloride in the urine are given at the bottom of the figure. Abscissae: time (approx.) after the test dose of water.

and 37, therefore, confirm the previous ones, and the conclusion is justified that dextrose is less active osmotically in causing a release of post-pituitary antidiuretic substance than is sodium chloride. It remains to attempt to throw some light on the events which underlie the response to the intravenous infusions of dextrose.

Attention has already been directed to the possibility that the small inhibitions resulting from the short-period intravenous injections of dextrose are conditioned by a release of insulin, and it now became of interest to determine the effects of infusing insulin over a period of 10 min.: they are illustrated in figure 38. During

the period *A*, insulin was infused at a rate of 0·3 unit/min. There is a definite inhibition of urine flow, but the flow has recovered completely before any signs of hypoglycaemia appear. At the beginning of *B* the animal began to look at its surroundings in an unusual way, and its increasing apprehension was accompanied by a fairly precipitous fall in urine flow, a fall which was, I imagine, of emotional origin. At *C*, when hypoglycaemic convulsions were imminent, a concentrated solution of 3g. dextrose (already prepared for this emergency) was rapidly injected
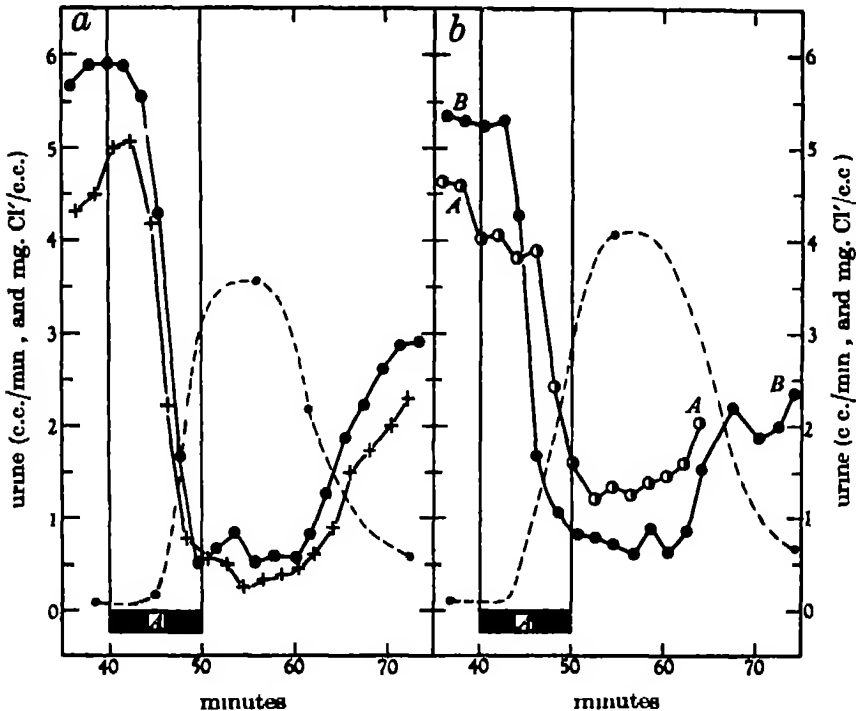
FIGURE 36. 'Nicky'. The responses to a 5 05 % increase in the osmotic pressure of the carotid blood produced by sodium chloride, and their assay. They should be compared with those (given in figure 37) to an equal increase produced by dextrose. The rectangles *A* cover the period of infusions. *a*, two responses to the intracarotid infusion of 0·6502M-NaCl at 1·65 c.c./min. *b*, responses to infusion of the same solution at the same rate but intravenously with post-pituitary extract 1·67 μU/sec. (graph *A*) and 3 33 μU/sec. (graph *B*). The course of the concentration of chloride in the urine during one of the responses in *a* and one of those in *b* is indicated by the broken-line graphs. Abscissae: time (approx.) after the test dose of water.

intravenously: the animal immediately regained its normal attitude. The recovery of urine flow just after the end of the infusion shows that the immediately preceding inhibition is not a direct result of a falling blood sugar, and it appears that insulin—if we accept the view that the inhibition is of pituitary origin—stimulates the neurohypophysis in a less indirect way to release its antidiuretic substance. Although this hypothesis would account satisfactorily for the inhibitions produced by the infusions of dextrose, the question as to whether or no the *difference* between the

response to the intracarotid and that to the intravenous infusion is of osmotic origin must remain an open one. If the response to the intracarotid infusion is solely of insulin origin, it follows that the secretion of insulin derives at least in part from the increase in the dextrose content of the carotid as distinct from the aortic blood. The question could seemingly be answered by recording the response of the kidney to an intracarotid and to an intravenous infusion of dextrose before and after denervation of the pancreas. Meantime, the conclusion to be drawn from
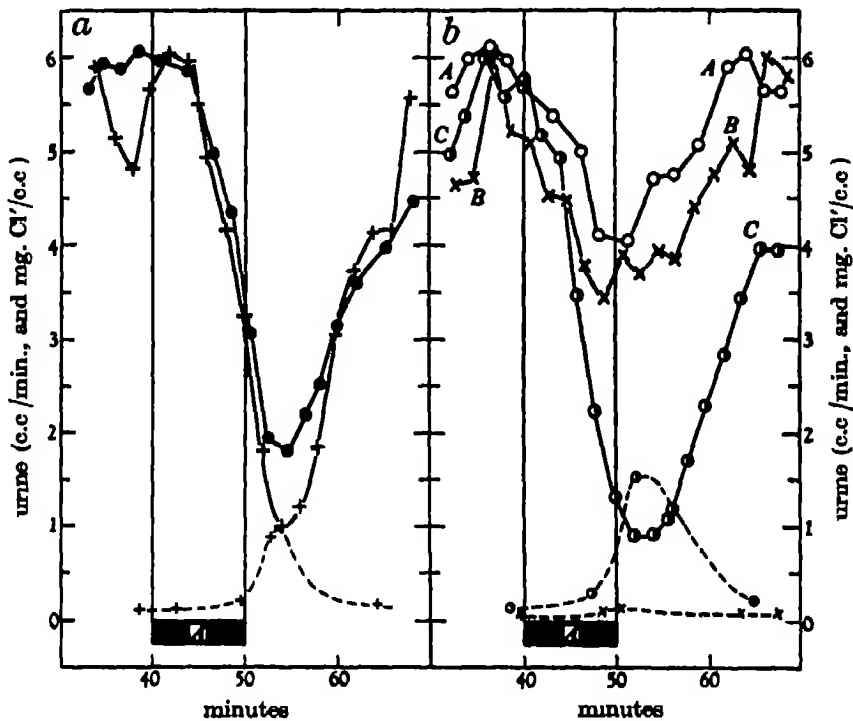
FIGURE 37. 'Nicky'. The responses to a 5·05% increase in the osmotic pressure of the carotid blood produced by dextrose, and their assay. They should be compared with those (given in figure 36) to an equal increase produced by sodium chloride. The rectangles A cover the period of the infusions. a, two responses to the intracarotid infusion of 1·166M-dextrose at 1·683 c.c./ min. b, responses to infusion of the same solution at the same rate but intravenously without (graphs A and B) and with (graph C) post-pituitary extract 1·67 μU/sec. The course of the concentration of chloride in the urine during one of the responses in a and two of those in b is indicated by the broken-line graphs. Abscissae: time (approx.) after the test dose of water.

these experiments is that when the osmoreceptors are exposed over a period of 10 min. to a rise in blood dextrose of some 250 mg./100 c.c., their 'membrane' is permeable to the dextrose: the experiments afford no evidence as to whether or no the permeability under these conditons is a complete one. We now turn to a description of comparable experiments with sucrose.

Comparison of the effects of osmotically comparable infusions of sodium chloride and sucrose. At about the same time as the experiments to which figure 36 relates were

made, when an increase of 5·05 % in the osmotic pressure of the carotid blood produced by infusions of sodium chloride was releasing post-pituitary antidiuretic substance at an equivalence of some 3·3 μU/sec., infusions of sucrose calculated to give the same increase in osmotic pressure caused about the same rate of release (see table 8). Later, the comparison was repeated, but under such conditions that a smaller increase is osmotic pressure was effected; and the results are given in figure 39, those on the left being the responses to infusions of sucrose, those on the right to infusions of sodium chloride. With the intracarotid infusions (graphs *A*)
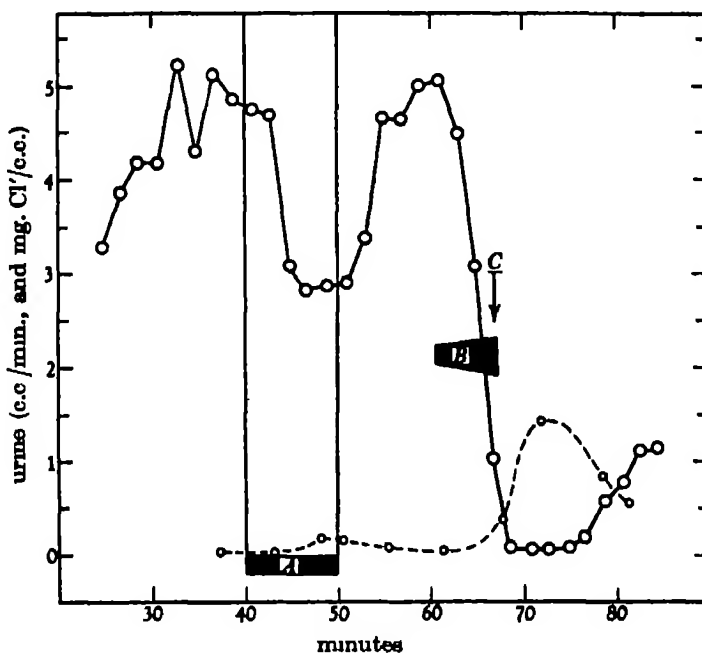
FIGURE 38. 'Nicky'. 28 March 1946. To illustrate the effects of an intravenous infusion of insulin over a period of 10 min. *A*, period of infusion. The solution infused was 0 81 % NaCl containing 6 units of insulin in 34 c.c., and the rate of infusion was 1·7 c.c./min. (0·3 unit/min.). *B*, manifestations of hypoglycaemia. At the arrow *C* 11 c.c. dextrose 27·45 g./100 c.c. were rapidly injected intravenously. The course of the concentration of chloride in the urine is indicated by the broken-line graph. Abscissae· time after the test dose of water.

the local increase in osmotic pressure is calculated to be 3·97 %. The graphs *C* show the responses to corresponding intravenous infusions when these contain post-pituitary extract in such amount that 1·67 μU/sec. are being administered: the responses to the intracarotid infusions are both a little larger, and that to the sucrose is possibly more so than that to the sodium chloride infusion, though quantitatively the resemblance between them is far more striking than the difference. The osmotic determination of the response to sodium chloride is thus confirmed.

TABLE 8. 'NICKY'. THE EFFECTS OF INFUSING SOLUTIONS OF SODIUM CHLORIDE, SODIUM SULPHATE, AND SUCROSE INTO THE RIGHT CAROTID ARTERY. EACH INFUSION PERIOD WAS 9 MIN. 52 SEC.

date	solute, strength (M) of solution and rate (c.c./min.) of infusion			percentage increase in osmotic pressure of carotid blood (calc.)	post-pituitary extract equivalent of response (μU/sec.)	remarks
	solute	(M)	(c.c./min.)			
29 Apr. 1946	sucrose	1·1353	1·62	5·05	3·3	—
27 May 1946	NaCl	0·6502	1·65	5·05	3·3	animal on heat
6 June 1946	NaCl	0·6502	1·65	5·05	3·3	animal on heat
28 Jan. 1947	NaCl	0·7736	1·05	3·97	2·0	—
4 Feb. 1947	sucrose	1·3512	1·05	3·97	2·2	—
2 Apr. 1947	Na₂SO₄	0·5844	1·05	3·96	2·2	—
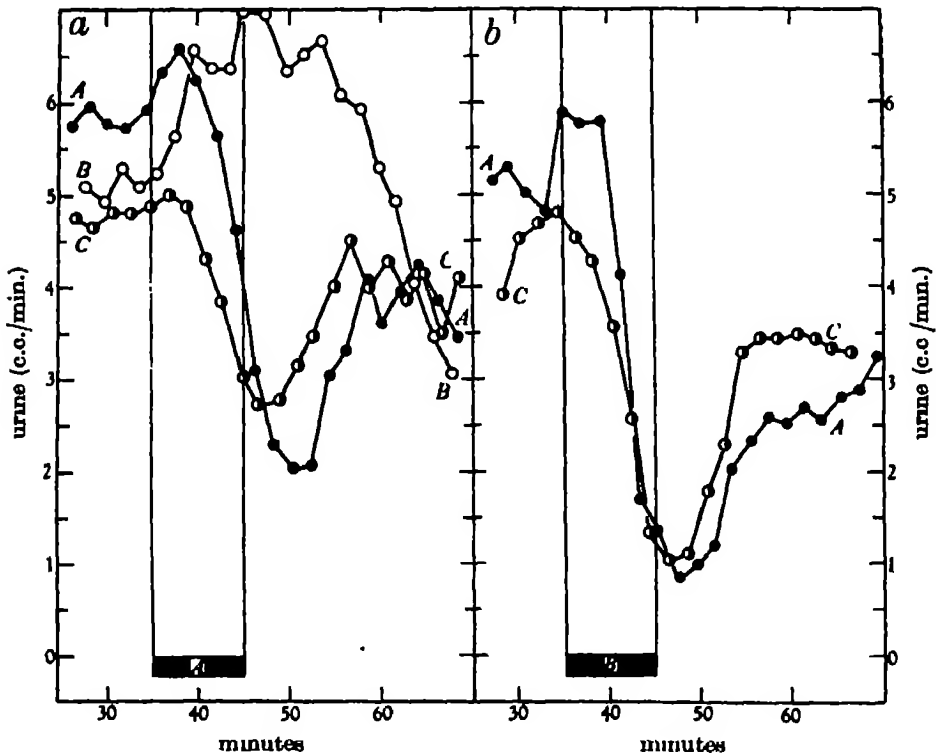
FIGURE 39. 'Nicky'. To illustrate the effects of osmotically comparable infusions of sucrose and sodium chloride. The rectangles A cover the period of the infusions. a, responses to 1·3512M-sucrose at 1·05 c.c./min. b, responses to 0·7736M-NaCl at 1·05 c.c /min. The graphs A give the responses to the intracarotid infusions, the graphs C those to the intravenous infusions with post-pituitary extract 1·67μU/sec. The graph B shows that no inhibition results from the intravenous infusion of sucrose alone. The experiments were made between 28 January and 5 February 1947. Abscissae: time (approx.) after the test dose of water.

The effects of infusions of sodium sulphate. Only one experiment of this nature has been performed, and the results are given in figure 40. In preparing the solution of sodium sulphate, the apparent degree of dissociation of the salt at its final molarity in the carotid blood (about 0·006) was taken to be 72 %, and the strength and rate of the intracarotid infusion were such that the calculated local increase in osmotic pressure would be the same (3·97 %) as in the two previous experiments with sodium chloride and sucrose (figure 39). The effect of such intracarotid infusion of sodium sulphate is shown in the graph *A* of figure 40. The graphs *C* and *D* give the responses to the same infusions but administered intravenously with post-pituitary extract at 1·67 and 3·33 μU/sec. respectively. The response to the intracarotid infusion lies between the two responses to the intravenous infusions, being nearer to the one containing the smaller amount of post-pituitary extract: it is judged to have an equivalence of 2·2 μU/sec., and to be indistinguishable from the responses elicited by osmotically comparable infusions of sodium chloride and sucrose.
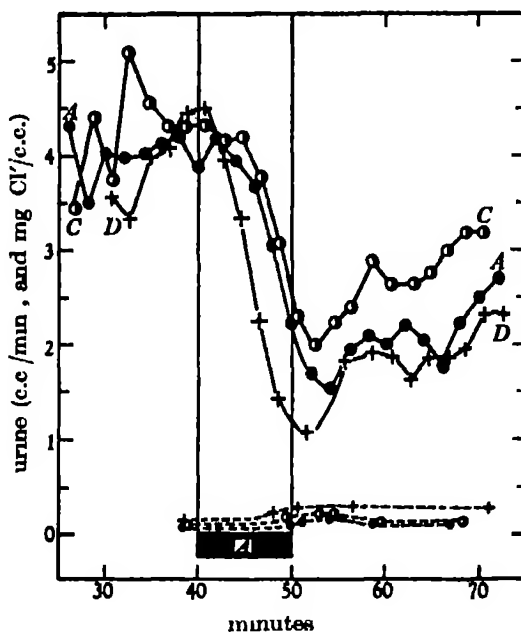
FIGURE 40. 'Nicky'. The effects of 10 min. infusions of Na_2SO_4 0·5844M at 1 05 c c./min. The graph *A* gives the response to an intracarotid infusion, the graphs *C* and *D* give the responses to intravenous infusions with post-pituitary extract 1·67 and 3·33 μU/sec. respectively. The courses of the urine chloride during these responses are indicated by the broken-line graphs. The rectangle *A* covers the period of the infusions. Abscissae. time (approx.) after the test dose of water.

The experiments so far described on the rates of release, in 'Nicky', of post-pituitary antidiuretic substance by increases in the osmotic pressure of the arterial blood between 50 and 4 % have shown that the former increase, operating over

a period of 10 sec. (see figure 30), is associated with an average rate of release of some 40μU/sec., the latter, operating over a period of 10 min. (table 8), with an average rate of release of some 2μU/sec. On the hypothesis that the maximum rate of urine flow during water diuresis is conditioned by an antecedent suppression of secretion of post-pituitary antidiuretic substance, and that this suppression results from a fall in the osmotic pressure of the *arterial* blood during the period of absorption of water from the small intestine, it would be of interest to follow the changes in osmotic pressure of the arterial blood during this period. As an indication—possibly an erroneous one—of such changes, the percentage (w/w) of
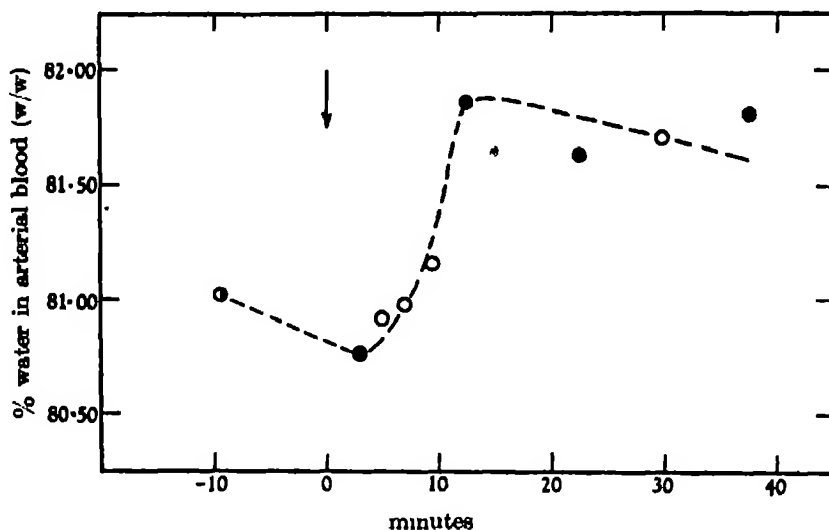
FIGURE 41. 'Jock'. Changes in the percentage (w/w) of water in the arterial blood (obtained by puncture of one of the carotid arterial loops) after the giving of 350 c.c. water by stomach tube. This, the test dose of water, was given at the arrow, the animal standing up while this was being done. Two experiments were made, and the initial values for the blood water (at −10·5 min.) were 81·15 and 80·90 %. The plot is the average of these, and corresponding adjustments have been made to the other values so as to make them comparable with the same initial value. Abscissae: time after the test dose of water.

water in the arterial blood has been followed after the administration of water by stomach tube, and the results are shown in figure 41. There is an increase of about 1 % within 12 min. of the giving of the water. If, therefore, there is a corresponding fall in osmotic pressure and this fall effectively suppresses the secretion of post-pituitary antidiuretic substance, one might expect that, during water diuresis, a prolonged *unilateral* intracarotid infusion calculated to maintain a local increase of 2 % in the osmotic pressure of the blood, would produce a course of antidiuresis which was roughly the inverse of that of diuresis following the test dose of water. With these considerations in mind we turn to a description of experiments in which such increase in osmotic pressure was maintained over a period of 40 min.

(8) *The effects of 40-minute exposure of the osmoreceptors to increases in the osmotic pressure of the carotid blood*

The effect of sodium chloride infusions. The results obtained with infusions of sodium chloride are illustrated in figure 42. The rectangle A covers the period (39·5 min.) of the infusions; and the solution used was 1·254 M, and it was given at a rate of 0·2725 c.c./min. With the intracarotid infusion (graph A) the urine flow, after a latent period of some 10 min., begins to fall, it reaches the low rate of
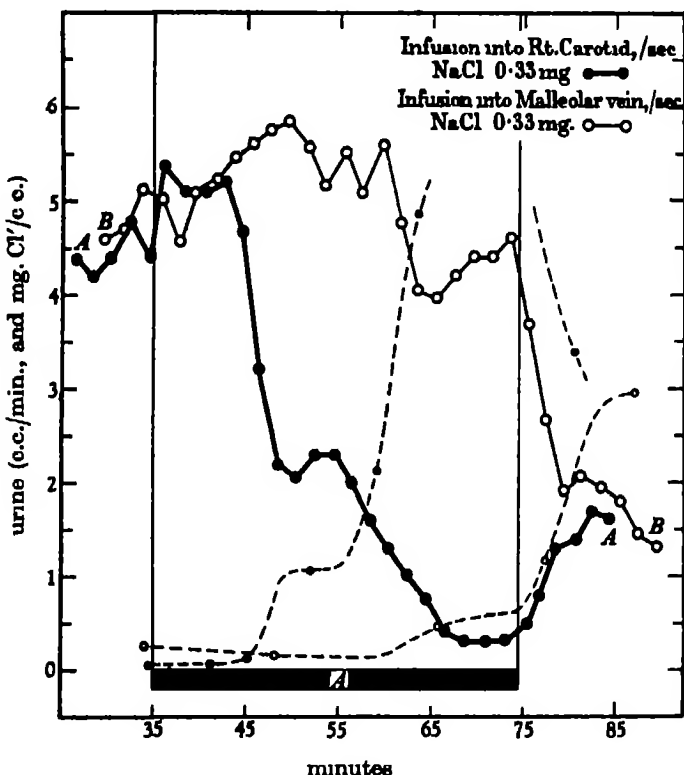
FIGURE 42. 'Nicky'. To illustrate the comparative effects of intracarotid (graph A) and intravenous (graph B) infusions of sodium chloride over a period of 39·5 min. With the intracarotid infusion the calculated local increase in osmotic pressure is 1·80%. The rectangle A covers the period of the infusions. The infusion was 1·254 M-NaCl at 0·2725 c.c./min. The courses of the concentration of chloride in the urine are indicated by the broken-line graphs. Abscissae: time (approx.) after the test dose of water.

0·3 c.c./min. some 20 min. later, and is maintained at about this level until the infusion is stopped. The urine flow then gradually increases. The intravenous infusion (graph B), on the other hand, has no appreciable influence on the course of the diuresis. The calculated percentage increase in the osmotic pressure of the carotid blood during the intra-arterial infusion is 1·80, corresponding with an increase in blood chloride of 9 mg./100 c.c.; and the experiment shows that under

these conditions the 'membrane' of the osmoreceptors maintains its relative or complete impermeability to sodium chloride for a period of at least 40 min. Six other and closely similar experiments have been made (table 9), and to the assay of the responses to the intracarotid infusions we shall return in a moment.

TABLE 9. 'NICKY'. THE EFFECTS OF INFUSING SOLUTIONS OF SODIUM CHLORIDE INTO THE RIGHT CAROTID ARTERY. EACH INFUSION PERIOD WAS 39·5 MIN.

date	strength (M) of NaCl solution, and rate (c.c./min.) of infusion		percentage increase in osmotic pressure of carotid blood (calc.)	post-pituitary extract equivalent of response (μU/sec.)
	(M)	(c.c./min.)		
May 1945	1·254*	0·2725	1·80	1
Sept. 1945	1·254†	0·2725	1·80	1
Jan. 1946	0·856	0·4110	1·77	1
Jan. 1946	0·856‡	0·4104	1·76	1
Sept. 1946	0·856	0·4070	1·73	0·5
Oct. 1946	0·856	0·3874	1·66	0·7
Oct. 1946	0·950	0·4082	1·98	0·8
Dec. 1946	1·340	0·2650	1·91	1
		Averages	1·81	0·9

* Illustrated in figure 46.   † Illustrated in figure 42.
‡ 1 mg. atropine sulph. s.c. 1 min. after test dose of water.

The effect of dextrose infusions. When, however, exactly comparable experiments are made with dextrose, a quite different result is seen. This is illustrated in figure 43. Two solutions of dextrose were used, 1·524 and 2·504M, the former being infused at 0·421, the latter at 0·2725 c.c./min.; and with the intracarotid infusions the calculated local increases in the osmotic pressure of the blood were 1·80 and 2·09 % respectively. The increase, therefore, was at least as great as in the experiment just described (figure 42) with sodium chloride. With dextrose, however, no difference was detected between the effects of the intracarotid and those of the intravenous infusions: in figure 43 the confused graphs to the right and above give the courses of urine flow during infusions into the carotid and into the malleolar vein. Now it will be shown later that when an increase in the osmotic pressure of the carotid blood of about 1·8 % is produced by an infusion of sodium chloride or of sucrose, post-pituitary antidiuretic substance is released at an equivalence of about 1 μU/sec. That this rate of osmotic release would be detectable, were it indeed occurring during the intracarotid infusions of dextrose, is shown by the graph (●—●) below and to the left in figure 43: this gives the response to a comparable intravenous infusion with the addition of post-pituitary extract 1 μU/sec. The conclusion is justified, therefore, that the 'membrane' of the osmoreceptors is permeable to dextrose to such degree that a rise in blood dextrose of some 90 mg / 100 c.c., a rise which incidentally is within the human pathological range, fails to elicit an antidiuretic secretory response by the neurohypophysis. Patients with diabetes mellitus are still permitted to be polyuric.

The effect of sucrose infusions. When comparable experiments are made with sucrose, the effects are very like those from sodium chloride. They are illustrated in figure 44. With the intracarotid infusion (graph *A*) the urine flow, after a latent period of some 5 min., begins to fall; it has reached a level of 0·75 c.c /min. by the time the infusion is stopped, and begins to recover towards the end of the experiment. The intravenous infusion (graph *B*), on the other hand, has no such anti-
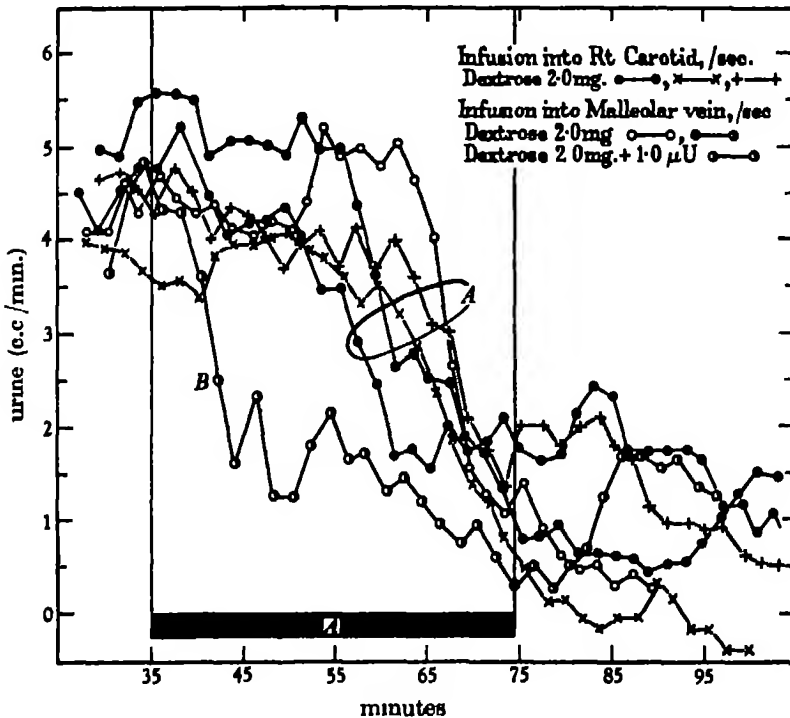
FIGURE 43. 'Nicky'. To illustrate the indistinguishability between the effects of intracarotid and intravenous infusions (graphs *A*) of dextrose over a period of 39·5 min. With the intracarotid infusions the calculated local increases in osmotic pressure are 1·80 and 2·09%. *B*, response to intravenous infusion + post-pituitary extract 1 μU/sec. The rectangle *A* covers the period of the infusions. The dextrose infusions were 1·524M at 0·421 c.c./min. (o-o, +-+, ●-●) and 2·504M at 0·2725 c.c./min. (⊙-⊙, ×-×, +-+). Abscissae: time (approx.) after the test dose of water.

diuretic action. Qualitatively, then, the results resemble those obtained with sodium chloride infusions; and quantitatively, as we shall see later, the response to the intracarotid infusion has a post-pituitary extract equivalence which is very close to that to such infusion of sodium chloride as produces in the carotid blood an osmotically identical effect.

In the experiment in which sucrose was infused into the carotid artery (figure 44, graph *A*) a little frothy nasal secretion appeared about 15 min. after the beginning of the infusion, and thereafter the animal cleared its nose occasionally. Although

this did not appear to disturb the animal unduly, it was thought wise to repeat the experiment after atropine had been given. Before doing so, however, the effects of the selected dose of atropine on this animal's blood pressure and pulse frequency were determined lest, during the period allotted on other occasions to infusion, such circulatory changes as might seriously affect the carotid blood flow, and therewith the local osmotic effects of the infusion, should still be evident. The results are
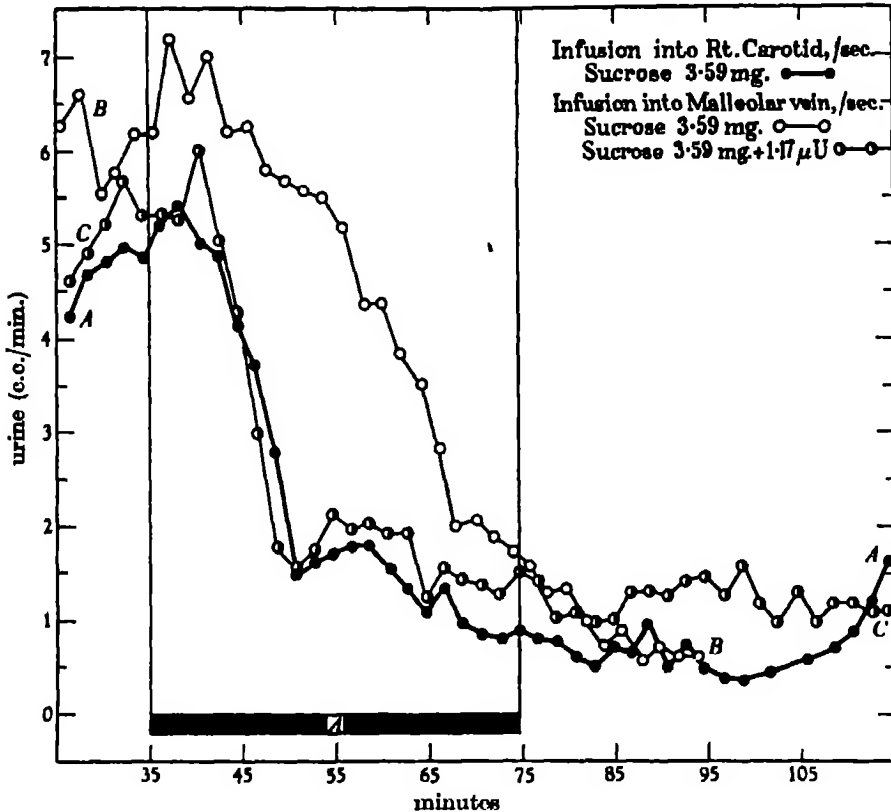
FIGURE 44. 'Nicky'. To illustrate the comparative effects of intracarotid (graph *A*) and intravenous (graph *B*) infusions of sucrose over a period of 39·5 min., and the assay of the response to the former. With the intracarotid infusion the calculated local increase in osmotic pressure is 1·80 %. The rectangle *A* covers the period of the infusions. The infusion was sucrose 1·485M at 0·424 c.c./min. The graph *C* gives the response to an intravenous infusion + postpituitary extract 1·17 μU/sec. Abscissae: time (approx.) after the test dose of water.

given in figure 45. The systolic blood pressure was measured by means of a cuff applied to the left carotid loop. The animal was given 1 mg. atropine sulphate by subcutaneous injection 1 min. after the test dose of water, and the effects of this on blood pressure and pulse frequency are shown by the graphs *A* and *B*. They reached their peak (185 mm. Hg and 186 beats/min.) at 17 min., and the pulse frequency then fell quite regularly to reach a steady state (95/min.) at 65 min. The blood pressure, however, fell more rapidly, so that by the time (35 min.) at which the

infusions were regularly begun in other experiments, it was within 10 mm. of its normal value. The experiment in which an intracarotid infusion of sucrose had been given was then repeated, but 1 mg. atropine sulphate was injected subcutaneously 1 min. after the test dose of water. On this occasion the animal was quite undisturbed; and the course of the urine flow was indistinguishable from that in the previous experiment and already illustrated in the graph *A* of figure 44. Details of these two intracarotid infusions and of their effects are given in table 10. The effects of comparable infusions of sodium sulphate have not yet been determined.
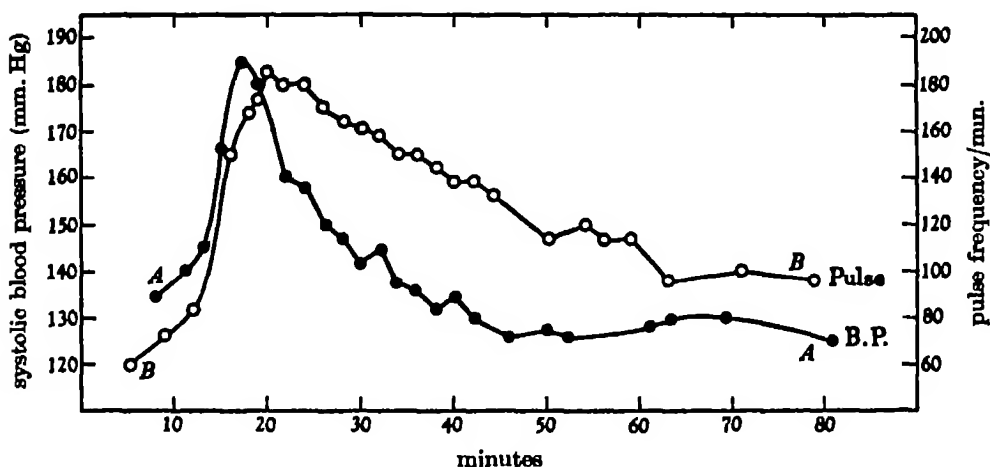
FIGURE 45. 'Nicky'. The courses of systolic blood pressure (*A*) and pulse frequency (*B*) after 1 mg. atropine sulphate subcutaneously at 1 min. Abscissae: time after the test dose of water.

TABLE 10. 'NICKY'. THE EFFECTS OF INFUSING SOLUTIONS OF SUCROSE INTO THE RIGHT CAROTID ARTERY. EACH INFUSION PERIOD WAS 39·5 MIN.

date	strength (M) of sucrose solution and rate (c.c./min.) of infusion		percentage increase in osmotic pressure of carotid blood (calc.)	post-pituitary extract equivalent of response (μU/sec.)
	(M)	(c.c./min.)		
Jan. 1946	1·485*	0·424	1·80	1·2
Feb. 1946	1·437†	0·421	1·73	1 2
		Averages	1 77	1 2

* Illustrated in figure 44.   † 1 mg. atropine sulph. s.c. 1 min. after the test dose of water.

In the former of the two experiments in table 10 it was observed that Benedict's test was positive when applied directly to the urine samples secreted from the eighth minute after the infusion had been started. The specimens gave, of course, a very strong reduction after hydrolysis. This presence of reducing sugar was confirmed in a comparable experiment in which the sucrose was infused intravenously with post-pituitary extract 1 μU/sec. Dr T. Mann very kindly determined

the total sugar and reducing sugar in the urine secreted during the last 14 min. of the infusion. Of the reducing sugar, part was fructose and the remainder was glucose. By the use of glucose oxidase, the enzyme which specifically oxidizes glucose, it was possible to determine the precise ratio of glucose to fructose. The urine (34 c.c.) was found to contain per c c. 35·75 mg. sucrose, and 3·25 mg. reducing sugar of which 0·75 mg. was glucose and 2·5 mg. fructose. In a third experiment, similar in every way to that represented in graph C of figure 44, blood samples were taken from a carotid artery 9 min. before the beginning of the infusion (sample 1), at the 25th minute of the infusion (sample 2) and 2 min. after the end of the infusion (sample 3). Specimens of urine (A, B and C), secreted at times corresponding with those at which the blood samples had been taken, were also retained for analysis. The results, for which I am greatly indebted to Dr Mann, are given in table 11. From these it is clear that when sucrose is infused into the dog, of the total sugar excreted by the kidney some 9 % is in a reducing form, and that some 20 % of this is glucose and the remainder presumably fructose. A certain proportion of the infused sucrose apparently undergoes inversion in the kidney.

TABLE 11. ANALYSES OF THE BLOOD AND URINE DURING AN EXPERIMENT ON 'NICKY' IN WHICH SUCROSE 1·487M WAS INFUSED INTRAVENOUSLY AT 0·425 C.C./MIN. FOR 39·5 MIN., THE INFUSION CONTAINING POST-PITUITARY EXTRACT 1·17μU/SEC. THE RESULTS ARE EXPRESSED IN MG./C.C.

blood				urine					
sample	reducing sugar, direct	reducing sugar after acid hydrolysis	'sucrose'	sample	total sugar	total reducing sugar	glucose	fructose	sucrose
1	1·08	1·11	0 03	A	0·198	0 189	—	—	--
2	1·15	1 98	0 83	B	27·40	2 49	0·42	2 07	24·91
3	0·84	1·66	0 82	C	60·00	5·10	1 05	4 05	54·90

(9) *The assay of the rate of release of post-pituitary antidiuretic substance during prolonged intracarotid infusions of sucrose and of sodium chloride*

It has already been shown that intracarotid infusions of sucrose and of sodium chloride associated with a local increase of 1·8 % in the osmotic pressure of the arterial blood, and operating over a period of 40 min , bring the urine flow gradually down to some 10 % of the maximum (figures 42 and 44). These responses have been assayed by incorporating in the corresponding intravenous infusion such quantity of post-pituitary extract as will give a response which closely matches that to the intracarotid infusion alone. This assay in the case of sucrose is illustrated in figure 44: the response to the intracarotid infusion (graph A) is fairly closely matched by that to the intravenous infusion when the latter contains post-pituitary extract in such amount that 1·17μU are being infused each second. A second experiment, in which

1 mg. atropine sulphate had been given subcutaneously 34 min. before the start of the intracarotid infusion (table 10) gave results which were closely similar. Exactly comparable experiments have been made with sodium chloride: the results are given in table 9, and the method of assay is illustrated in figure 46. The response to the intracarotid infusion (●—●) lies, for most of its course, between the responses to 0·67 ((◑—◑) and 1·17 (o—o) μU/sec., being nearer to and probably a little
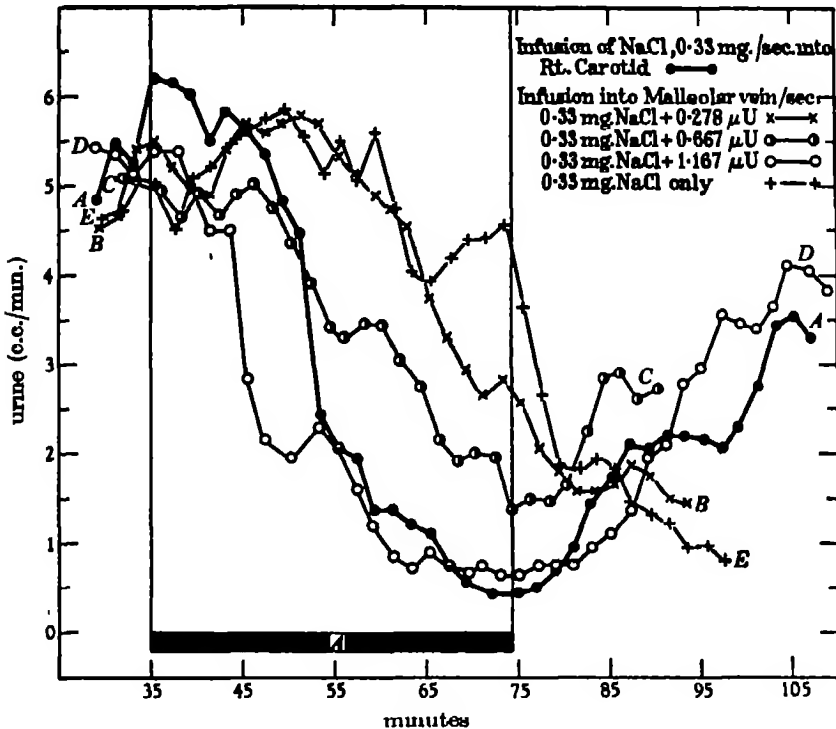
FIGURE 46. 'Nicky'. To illustrate the assay of a response to an intracarotid infusion of sodium chloride. With the intracarotid infusion the calculated local increase in osmotic pressure is 1·80 %. The infusions were 1·254 M-NaCl at 0·2725 c.c./min.; and the rectangle A covers their period (39·5 min.). Graph A (●—●), intracarotid infusion, graph E (+—+), intravenous infusion. The remaining graphs (B, ×—×; C, ◑—◑; D, o—o) give the responses to the intravenous infusion with post-pituitary extract 0·28, 0·67 and 1·17 μU/sec. Abscissae: time (approx.) after the test dose of water.

less than the latter: it is assayed as having a post-pituitary extract equivalence of 1 μU/sec. The results of all such experiments are given in table 9; and although it would appear from these, as from those of the 10 min. infusions, that for a given increase in the osmotic pressure of the carotid blood, sodium chloride is a little less active than sucrose, this in no way discredits the theory of their essentially osmotic determination: on the contrary, the assays are so nearly identical as to give the theory firm support.

D. *The hormonal characterization of post-pituitary antidiuretic substance*

Three conclusions follow from the results of the 40 min. infusions. First, that increases in the osmotic pressure of the arterial blood—increases which, when large, were shown by the short-period injections to release post-pituitary anti-diuretic substance—are still operative when they are reduced to values well within a range which may reasonably be regarded as physiological. In 'Nicky' an increase of only 1·8 % in the osmotic pressure of the carotid blood gradually reduces the rate of urine flow from a water-diuresis maximum to the sort of rate which prevails at the beginning and end of a normal response to ingested water, i.e. a reduction of some 90 %. The smallness of the osmotic pressure increase gains additional interest when it is recollected that the carotid infusions were unilateral. For if, as is probable, only half of the total number of osmoreceptors are being exposed under these conditions to the osmotic pressure increment, an increase of some 1 % only (54 mm. Hg) in the osmotic pressure of the *aortic* blood would suffice to produce the same degree of inhibition, i.e. a reduction to some 10 % of the maximum rate of which the kidney is capable during water diuresis. Secondly, the results of the 40 min. infusions demonstrate that the induced change in the osmotic pressure of the arterial blood which is responsible for this degree of reduction in urine flow, itself causes the release of post-pituitary antidiuretic substance at an average rate of $1\mu U/sec.$ (0.5×10^{-9} g/sec. in terms of the standard powder), this being the intermediating agency through which the change in osmotic pressure becomes effective. Thirdly, the recovery of urine flow when the intracarotid infusion is stopped (figure 46) shows that the secretion of post-pituitary antidiuretic substance is now inhibited by the local fall in osmotic pressure and consequent depression of activity in the osmoreceptors, the progression of this recovery being attributable to the gradual destruction in the kidney and, maybe, in the blood, of the quantity of antidiuretic substance which was maintaining the secretion of urine at a non-diuretic level. The latent period between the peak of the water-load curve and the maximum rate of urine secretion, to which I referred earlier and promised to return, is clearly to be attributed to the same process, as indeed was envisaged at the time that work was done. Water diuresis, then, is fitly and accurately described as a condition of physiological diabetes insipidus, and there can be little doubt that the antidiuretic secretion of the neurohypophysis is a hormone in the physiological sense that its liberation is mainly and continually governed by the contemporary concentration of sodium chloride in the carotid arterial plasma. The physiological fitness of this control is emphasized by its quantitative aspects, in that changes within the range and of the order of 1 % in the osmotic pressure of the arterial blood lead, through the intermediation of the antidiuretic hormone, to changes in the rate of water excretion within the range and of the order of 1000 %: the maintenance of near constancy in the osmotic pressure of the internal environment is thereby achieved.

IV. DISCUSSION

The work reviewed and described here has demonstrated that post-pituitary antidiuretic substance is released in the living animal by two distinct agencies, emotional stress and an increase in the osmotic pressure of the arterial blood. The release under the former condition is doubtless owing to eventual stimulation of the cytons of the supraoptic, and possibly of the paraventricular nuclei, whose axons pass down the stalk to the pars nervosa. On the assumption that the antidiuretic substance in post-pituitary extract has, on reaching the kidney, the same structural form as has that released endogenously, this release is shown to be inhibited or suppressed by an increase in sympathetic activity associated with the animal's discomposure, and by an intravenous injection of adrenaline or tyramine 30 sec. before the application of an appropriate stimulus. The evidence is against the sympathetic or sympatheticomimetic block's being a function of the pressor response to these agents or of contingent changes in cerebral blood flow, and in favour, therefore, of its being the result of a direct action of adrenaline or tyramine on some process in the chain of events in the central nervous system initiated by the stimulus, and culminating in the release of post-pituitary antidiuretic substance. Adrenaline is extremely active in this regard in the sympathectomized animal, its effect being demonstrable when the drug is injected intravenously in a dosage of $5\mu g$; but there is at present no evidence as to the precise site and mode of its action. It may be that it is effective, too, in preventing the inhibition of water diuresis by small (some 6 % of the blood volume) arterial haemorrhages which, as Rydin and I showed, cause an inhibition of the pituitary type in dogs in which the splanchnic nerves have been divided with or without removal of the abdominal sympathetic chains. It would be of interest to know whether this inhibition is indeed dependent on the presence of the pars nervosa, and whether such arterial haemorrhage is ineffective or less effective in this regard in animals in which the sympathetic system is intact. Although the release of post-pituitary antidiuretic substance by emotional stress is facilitated after sympathectomy, this is apparently not so for the osmotic release of this substance. Inquiry has not as yet been specifically directed to this question, but the few responses which were obtained from 'Nicky' before splanchnic section and partial abdominal sympathectomy, did not differ impressively from those to similar intracarotid injections before this operation. Moreover, in the case of another animal ('Alice') I have plotted all the results of intracarotid injections before and after splanchnic section in the manner of those shown in figure 29: both sets of results were fairly evenly distributed over a wide range of release, and the plotted relations in all of the eight experiments before operation lay to the left of a line joining those of the five experiments after operation. And if comparison is made between the results (figure 29) obtained from 'Sally' (intact sympathetic system) and those (figure 30) from 'Nicky' (sympathectomized), it will be seen that the latter animal is certainly not more sensitive than the former to a given osmotic stimulus. This, of course, is not to say that

were the adrenaline content of the blood of the non-sympathectomized animal raised, the response to a given osmotic stimulus would be unaffected; though it is not clear how an appropriate test of this could be made in the living animal. I mention these findings lest doubt should have been felt as to the justification of using the later results from 'Nicky' as data for the quantitative assessment of their physiological import.

In connexion with the inhibition of water diuresis by intracarotid injections of hypertonic solutions, little remains to be said. The post-pituitary origin of the inhibition has been demonstrated; and the short-period injections have shown that the factor which is operative in eliciting the response is the increase in the osmotic pressure of the carotid blood. This factor, as is disclosed by the results of the long-period (40 min.) infusions of sodium chloride and of sucrose, is still operative within the compass of physiological degrees of change. Osmoreceptors, therefore, exist: these are continually engaged in transmuting osmotic pressure changes in their environment into appropriately effective messages to the neurohypophysis; and the justification for the intellectual apprehension of these receptors gives the promise of their eventual structural identification. The befitting sensitivity and functional propriety of this form of control of the renal excretion of water, and the maintenance thereby of near constancy in the osmotic pressure of the animal's internal environment, have already been emphasized: one can scarcely conceive an arrangement more elegant in apparent design, or more indicative in purport, in an animal order whose developmental secession from intimacy with the external environment has depended upon the concurrent acquisition of control over the internal environment.

There is one small difference and one small similarity, which have not hitherto been mentioned, between the responses to the intracarotid infusions of sodium chloride and their matches by intravenous infusion of post-pituitary extract. In the latter, the rate of urine flow begins to decline a little sooner than in the former (see figures 39 and 46): seemingly the osmotic release reaches its final value somewhat gradually (see also figure 31). The similarity alluded to just now is the presence in the responses to both intracarotid and intravenous infusions over a 40 min. period, of a transient interruption in their course (figures 42, 44 and 46). This interruption, therefore, is of renal origin: it is often seen during the subsidence of simple responses to ingested water.

It will be asked why the immediate reaction of the osmoreceptors to a *fall* in the osmotic pressure of their environment has not been investigated. The reason is that with the short-period injections and the 10 min. infusions, there is no theoretical justification for the expectation that so transient a suppression of activity in the receptors would evoke a significant increase in the rate of urine secretion, and that with the 40 min. intracarotid infusions a local fall of 2 % in the osmotic pressure of the arterial blood could be sustained only by the unilateral infusion of distilled water at a rate of some 2 c.c./min. Even under these conditions no diuresis is to be expected, seeing that a proportion of the receptors are not then exposed to the

induced fall in osmotic pressure; and if indeed an increase in the rate of urine flow occurred, the rate of water infusion would itself obtrude in the resolution of its cause. But the demonstration that the osmoreceptors lie in the vascular bed of the internal carotid artery, the sectional area of whose lumen is, in the dog, only some 25% of that of the common carotid, now gives the possibility of developing a suitable technique for such investigation. For if the external carotids could in some way be disjoined from the common carotid trunks *without upsetting the balance of arterial inflows into the Circle of Willis*, a theoretically effective lowering of the osmotic pressure of the internal carotid blood would then be realized by infusing into the common carotid trunks, or into one of them after destruction of the contralateral supraoptic nucleus, a volume of water much smaller than that otherwise needed.

A further and collateral investigation which could be profitably undertaken with suitable extensions of the techniques described above, and one which has probably occurred to the reader already, is the test of whether, with the release of the antidiuretic hormone by the physiological stimulus of osmotic-pressure increase, there is an obligatory release of oxytocic substance from the pars nervosa.

The enquiry into the effects of injecting and infusing 'hypertonic' solutions into the carotid artery, in disclosing the osmotic basis of the responses, has at the same time shown that the 'membrane' of the osmoreceptors possesses the property of specific permeability. The 'membrane' is freely permeable to urea. It is probably less freely so to dextrose, since it would seem very unlikely that most if not the whole difference between the responses to intracarotid and intravenous infusions of dextrose over a period of 10 min., and *a fortiori* between the responses to the short-period injections, derives from some mode of action other than an osmotic one. The possibility of there being a larger release of insulin from an intracarotid than from a similar intravenous injection or infusion of dextrose, can be assessed only by determining whether or no denervation of the pancreas affects the difference between the renal responses to such injection or infusion. The fact, however, that the response to an intracarotid infusion of dextrose over a 10 min period has a smaller post-pituitary extract equivalence than that to a comparable infusion of sodium chloride, shows that the 'membrane' of the osmoreceptors is at least partially permeable to dextrose. Moreover, with the 40 min. intracarotid infusions of dextrose, producing a local increase of some 2% in the osmotic pressure of the blood, the permeability is such that no release of the antidiuretic hormone is detectable. To sodium chloride, sucrose, and sodium sulphate, on the other hand, the 'membrane' is relatively impermeable as judged by the close correspondence between their osmotic effects within the compass of a 10 min. period, and, in the case of sodium chloride and sucrose, of a 40 min. period. Whether the osmoreceptors accommodate during longer periods of exposure to a constant increase in the sodium chloride content of the carotid blood, whether, that is to say, their 'membrane' under such conditions becomes partially permeable to sodium chloride, is not yet known: it would seem highly probable that this is so, in view of the fact

that water diuresis may be elicited when the body is still retaining a large proportion of previously ingested salt (Baird & Haldane 1922; Baldes & Smirk 1934). Their accommodation to a lowered osmotic pressure, too, would seem likely, seeing that when the human subject is placed on a mineral-poor diet for 7 or 8 days no state of diuresis is thereby induced, but the ingestion of water then causes a diuresis, albeit a smaller one than in the same subject when on a normal diet (Baldes & Smirk 1934). The attribution to the osmoreceptors of the property of slow accommodation to their environment of sodium chloride, gives a fitting and simple interpretation to the results of these experiments.

Finally, we come to consider the localization and histological characters of the osmoreceptors. The persistence of the similarity in the responses to like injections into the two common carotid arteries after section of one carotid nerve ('Alice', number 307), belied the attractive hypothesis that the carotid body, innervated by the glossopharyngeal nerve through its carotid branch, was engaged in 'tasting' the osmotic properties of the internal environment. The receptors lie, as the experiments with 'Pat' and 'Sally' have demonstrated, somewhere in the vascular bed normally supplied by the internal carotid artery. they share with the brain itself, therefore, the high guarantee which the Circle of Willis gives, of a blood supply both resourceful and relatively unvarying—a seemingly not unimportant attribute for structures with so fundamental a function. Their linkage with the neurohypophysis justifies a careful histological scrutiny of its main nucleus. Although such an investigation has not as yet been carried far, I have on occasion observed within the field of the supraoptic nucleus, vesicles with features which appear to be admirably suited to an osmoreceptive function. These bodies are illustrated in figure 47, plate 11. In each of four dogs which were anaesthetized with chloralose, and their brains thereupon fixed by perfusion with formaldehyde-saline at room temperature, these structures have been identified in both divisions of the supraoptic nucleus, whereas in one animal which had died some hours before the pituitary region was exsected and fixed in formaldehyde-saline, none were seen. this is evidence against their being artefacts. In one of the above four animals ('Jetsam', figure 10, plate 9) serial sections of the whole supraoptic region were made, and those of the nuclei were drawn at a magnification of 87 ×. The roughly spherical nature of the vesicles was thereby disclosed; and their maximum anteroposterior and transverse diameters were computed. There were eighty-nine such vesicles: their distribution among the anterior and posterior divisions of the supraoptic nuclei is given in table 12, and the frequency distribution of their anteroposterior and transverse diameters is shown in figure 48. This animal ('Jetsam') weighed 5 kg. In another and much larger animal ('Sally', 21 kg.) whose posterior lobe had been removed 51 days before fixation of the brain, only thirty-two vesicles were counted. The vesicles do not contain fat. Are they indeed tiny osmometers to the surface of which the dendrites of some of the supraoptic cytons are attached as stretch receptors, increases in the osmotic pressure of their highly vascular environment (Finley 1940) being thereby transmuted into electrical wave

messages to the secretory endings of the supraoptico-hypophysial tracts? The average of the means of the antero-posterior and transverse diameters of 'Jetsam's' vesicles (table 12) is $52 \cdot 5\mu$, which on correction for contraction in the preparation of paraffin-embedded tissue (factor $= 1 \cdot 3$) becomes 68μ, and calculation shows that
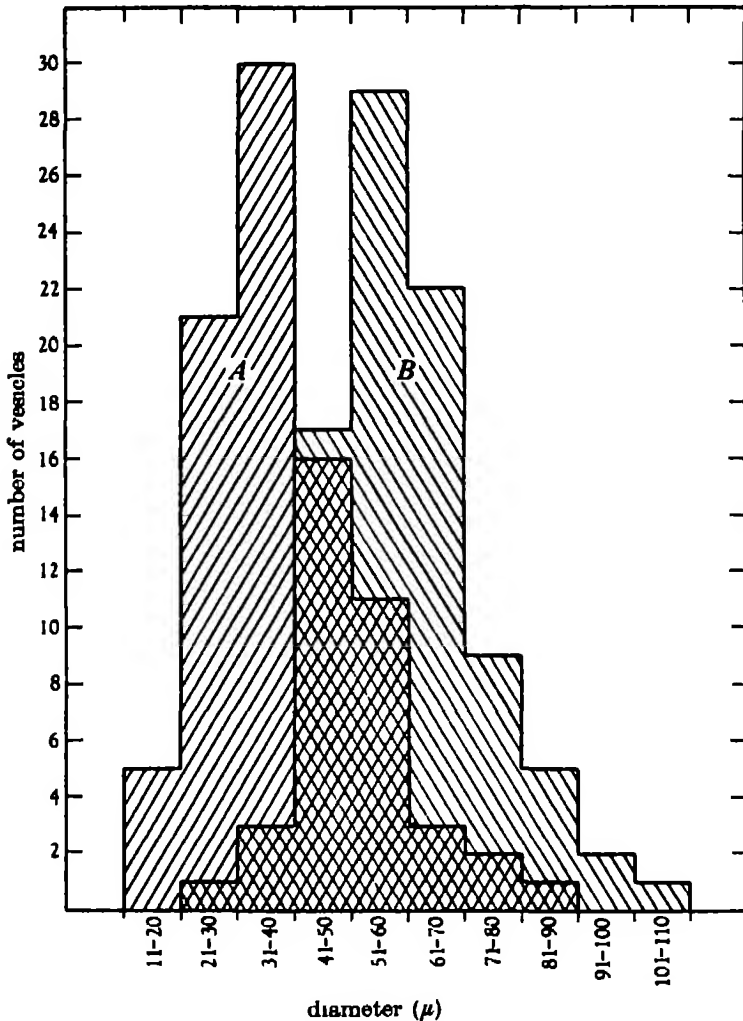
FIGURE 48. 'Jetsam'. The frequency distribution of the antero-posterior (A) and transverse (B) diameters of the vesicles in the supraoptic nuclei.

were a spherical osmometer of this size exposed to an increase in osmotic pressure of 1%, the shortening of its radius would be 118 mμ. The total surface area of 'Jetsam's' vesicles is computed to be 1·3 mm.². I would emphasize, however, that the ascription of an osmotic function to these structures is at present little more than a speculative indulgence. Nevertheless, there is, I feel, both fascination and

challenge in the thought that the dog's acquisition and tenure of its place in Nature should derive from the permeability properties of a colony of membranes, the aggregate of whose unilateral surfaces occupies an area of the order of 1 mm.^{2}.

TABLE 12. 'JETSAM', WT. 5 KG. NUMBER OF VESICLES IN THE SUPRAOPTIC NUCLEI AND THEIR DIAMETERS IN μ

| | right nucleus | | left nucleus | | total number of vesicles and their average diameter |
	anterior division	posterior division	anterior division	posterior division	
number of vesicles	11	30	16	3½	89
mean antero-posterior diameter	47	44	43	42	44
mean transverse diameter	55	63	57	62	61

Mr G. S. Adair, F.R.S., gave much help and advice in the calculations of osmotic pressure changes, and Dr J. N. Agar kindly supplied certain data in connexion with the apparent degrees of dissociation of sodium sulphate in blood; to them I am most grateful. I would also express my gratitude to Dr T. Mann for the analyses of the blood and urine in the sucrose-infusion experiments, to Dr M. Karvonen for much help in connexion with the estimations of carotid blood flow, and to Dr H. Konzett for his help with the intracarotid injections of Evans's blue. The microphotographs in figure 47 were made by Mr J. A. F. Fozzard in the Department of Anatomy, by arrangement with Professor H. A. Harris; to them I am much indebted. Mr A. Hogwood has given technical assistance throughout this work, and his efficient help is gratefully acknowledged.

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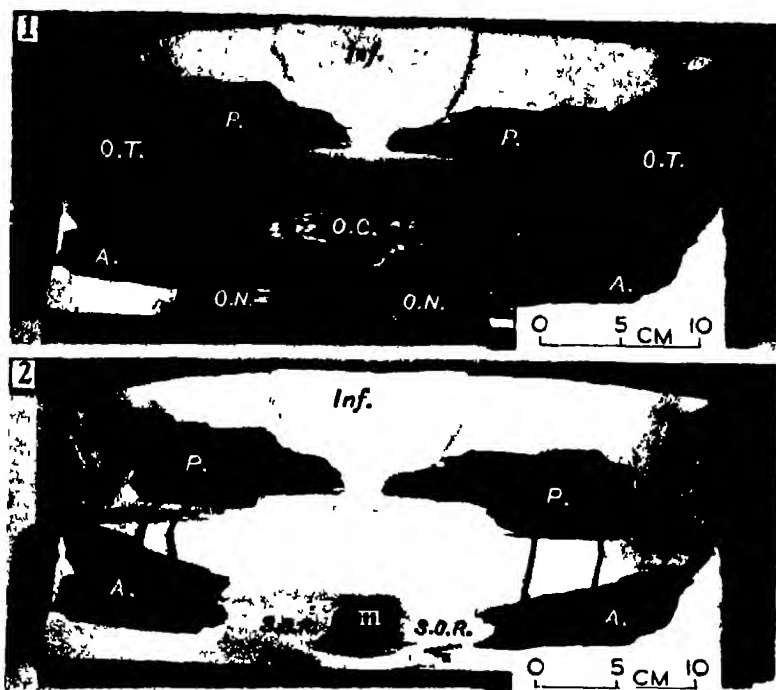
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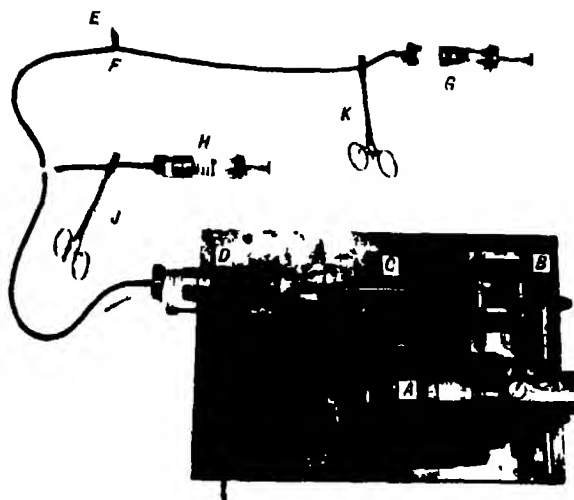
Figure 11

FIGURE 12
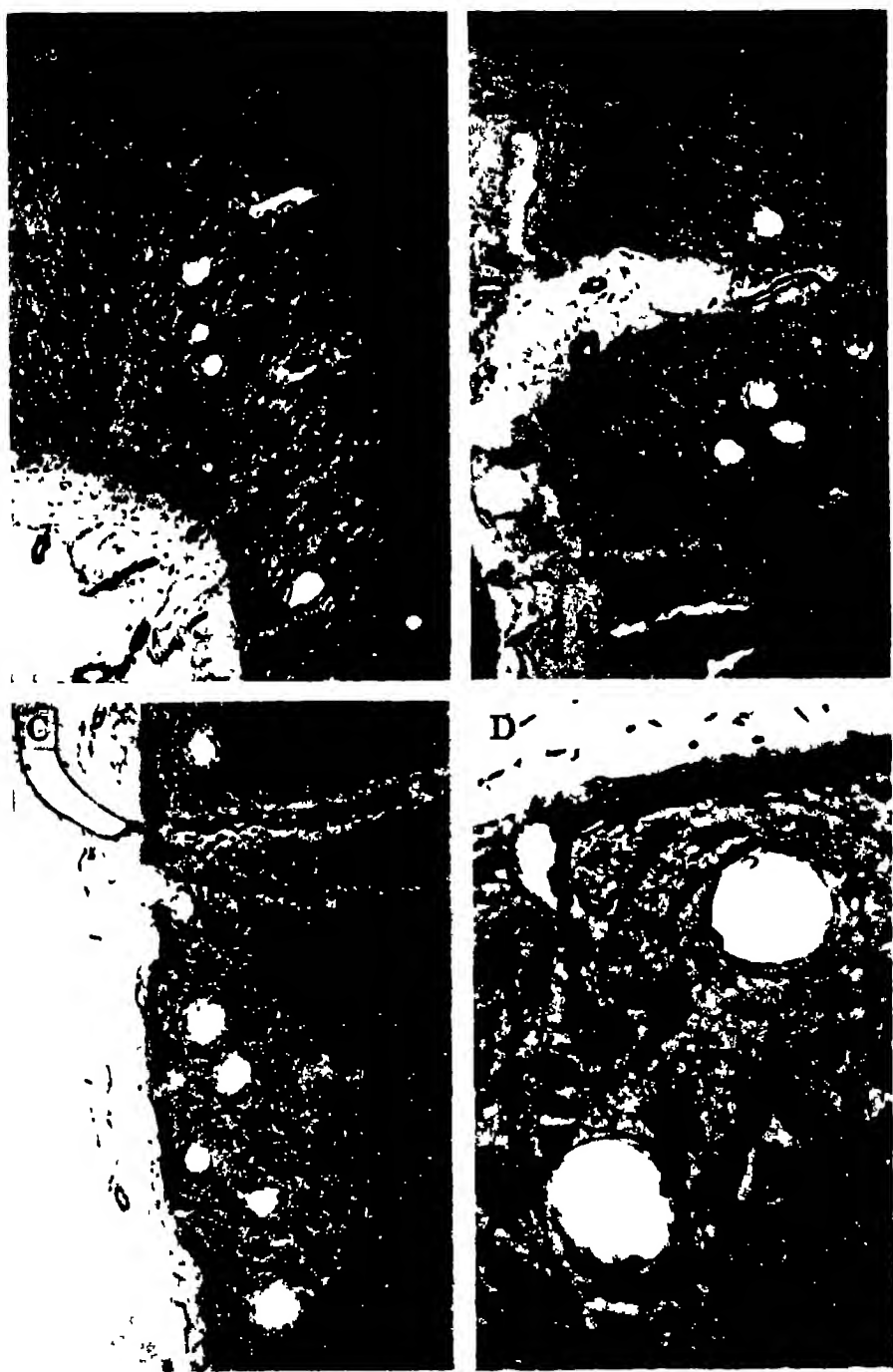
FIGURE 13

FIGURE 47

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FIGURE 10. 'Jetsam', 5·0 kg. Reconstruction (× 50 linear) of prepituitary region of brain. Photographs of ventral surface; with the optic nerves, chiasma and tracts *in situ* (1), and removed (2). *A*, anterior divisions; *P*, posterior divisions of supraoptic nucleus. *Inf*, infundibulum. *ON*, optic nerves. *OC*, optic chiasma. *OT*, optic tracts. III, 3rd ventricle. *SOR*, supraoptic recess of 3rd ventricle. In the model, the anterior and posterior divisions of the nuclei are joined by wires.

FIGURE 11 Apparatus for long-period intracarotid and intravenous infusions. *A*, constant speed motor. *B*, reduction gearing. *C*, micrometer screw. *D*, syringe containing the test solution. *E*, infusion needle. *F*, glass T-piece. *G*, syringe containing NaCl 0·85%. *H*, syringe containing the same solution as *D*. *J*, *K*, clamps.

FIGURE 12. 'Nicky', 17 May 1945. A piece of cotton-wool has been put behind the carotid loops. Most of the injection and infusion experiments were made on this animal.

FIGURE 13. 'Pat', June 1945. A piece of black paper has been put behind the carotid loops. The effects of tying the left internal carotid artery on the responses to intracarotid injections of hypertonic solutions of sodium chloride in this animal are illustrated in figure 26.

FIGURE 47. Photomicrographs of 'Jetsam's' supraoptic nuclei. Frontal sections 10μ thick; haematoxylin and eosin. To show the vesicles referred to in the discussion. *A*, anterior division of left; and *B*, anterior division of right nucleus (× 67·5). *C*, posterior division of left nucleus (× 67·5). *D*, posterior division of left nucleus (× 315).

Glutinosin: a fungistatic metabolic product of the mould *Metarrhizium glutinosum* S. Pope

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Culture media on which the fungus *Metarrhizium glutinosum* has grown are highly toxic to other fungi. The fungus grows well on such standard synthetic media as Czapek-Dox or Raulin-Thom, but use of a crude grade of glucose instead of pure dextrose encourages sporulation and slightly increases the fungistatic activity of culture filtrates It has been shown that yeast extract, biotin and aneurin supplements to a pure dextrose medium produce a similar response

In a study of the nature of the nitrogen source in the culture medium in relation to production of fungistatic culture filtrates, it has been found that nitrate nitrogen is very effective for growth of the fungus but not very effective for production of the fungistatic substance. Inorganic ammonium salts are ineffective in both respects, but peptone or ammonium tartrate were found to be highly effective both for growth and production of the fungistatic substance.

This superiority of ammonium tartrate led to an investigation of the relation between organic acids and ammonia assimilation. Addition of a variety of organic acids, in concentrations of 0 05 to 1 0 %, to an ammonium sulphate medium, led to great increases in growth and in production of the fungistatic substance. These acids included tartaric, malic, succinic, malonic, oxalic, citric, acetic, glycollic, pyruvic, aspartic and glutamic acids These acids do not stimulate when added to a nitrate, amino-acid or peptone medium Various possible explanations of this effect have been considered, and it is suggested that *M. glutinosum* is unable, when nitrogen is supplied as an ammonium salt, to break down glucose to the 3-, 4- or 5-carbon acids which normally enter into the transformations of the Krebs cycle. Accordingly, this fungus is unable to assimilate ammonia, which is probably normally, in fungi as in other plants, condensed with α-keto acids, produced by carbohydrate degradation, to form corresponding α-amino-acids. When these acids, or acids which could probably be transformed into such acids in the cell, are supplied in the nutrient medium, growth is accordingly increased.

The active fungistatic material, glutinosin, can be extracted from culture filtrates by extraction with ether, petrol-ether or benzene, the latter being most effective, more effective still is treatment of the culture filtrate with charcoal followed by elution of the charcoal with hot benzene On evaporation of the solvent and recrystallization of the residue from ethyl alcohol, glutinosin is obtained in pure crystalline form, analyses and molecular weight determinations indicate that glutinosin has the molecular formula $C_{18}H_{60}O_{19}$.

Glutinosin is highly toxic to many fungi but is markedly specific, germination of spores of *Botrytis allii* in Czapek-Dox is inhibited by $0 8 \mu g /ml$, but *Trichoderma viride* is not inhibited by $50 \mu g /ml$ It is at most only slightly toxic to bacteria. Aqueous solutions are relatively stable, even when heated, but are less stable when alkaline than when acid

A volatile metabolic product of *Metarrhizium glutinosum* causes a severe dermatitis, the dermatitic substance is extracted by the same organic solvents as used for the extraction of glutinosin, but it is not identical with glutinosin.

INTRODUCTION

Metarrhizium glutinosum S. Pope is a recently described species (Pope 1944) which has been isolated in the United States from deteriorated baled cotton and from Maryland soil. This fungus has been shown by Greathouse, Klemme & Barker (1942) to cause very rapid decomposition of cellulose, it will grow vigorously on cellulose, if the necessary mineral salts are present, without any additional source of carbon.

The genus *Metarrhizium* has been regarded as being typically parasitic on insects. The best known species, the green muscardine (*M anisopliae* (Metsch.) Sorok.), has been widely used in experimental work on biological control of various insect pests of agricultural crops. Thom (1930) has pointed out that organisms apparently belonging to this genus are frequently isolated from soils under conditions suggesting that they must be capable of existing independently as saprophytes. *M. glutinosum* appears to be such a species, and the genus *Metarrhizium* must now be regarded as composed partly of entomophagous species and partly of saprophytes. *M. glutinosum* has only been recorded from the two sources mentioned above, but, in view of Thom's remark concerning the frequency of isolation of *Metarrhizium* spp. from soil, it may be a commoner species than would at first seem to be the case.

In a preliminary account (Brian & McGowan 1946) a metabolic product of *M. glutinosum* toxic to other fungi has been described, for which the name glutinosin was proposed. The present paper describes, in greater detail, conditions affecting the production of glutinosin, methods of extraction of pure glutinosin from culture filtrates and its range of biological activity.

EXPERIMENTAL METHODS

Assays of fungistatic and bacteriostatic potency

For routine assays of fungistatic activity of culture filtrates, a spore germination test with conidia of *Botrytis allii* Munn. has been used. This has been described in detail previously (Brian & Hemming 1945). Assays of bacteriostatic activity have been made by the conventional serial dilution technique in broth cultures. Where other special techniques have been used they are described in the text below.

Methods of culture

In experiments not involving extraction of the active material, cultures have either been on 30 ml. of medium in 100 ml. pyrex conical flasks or on 250 ml. of medium in glass 'Glaxo' culture vessels (Clayton, Hems, Robinson, Andrews & Hunwicke 1944). In the case of experiments with 100 ml. flasks, the practice has been to set up a large number of flask cultures with each medium, five flasks being selected at random whenever an assay was required, the contents of the five flasks being bulked, the mycelium filtered off and a sample of the filtrate taken for assay. In experiments with 'Glaxo' culture vessels, six vessels were set up with each medium, samples of culture liquor taken periodically from each vessel with a sterile pipette and the samples from each set of six vessels bulked for assay.

For bulk production earthenware culture vessels, each holding 1 l. of medium, have been used.

All cultures have been incubated at 25° C in temperature-controlled incubator rooms.

Production of spores for inoculum

Metarrhizium glutinosum sporulates slowly and sparsely on mineral salt-glucose media such as Czapek-Dox agar or Raulin-Thom agar. Sporulation is rather better on 2 % malt-extract agar or 5 % malt-extract agar. Sporulation is greatly improved by addition of yeast extract The medium finally standardized for production of spores was 5 % malt-extract agar supplemented by 1 % 'Difco' yeast extract; on this medium sporulation was abundant after 5 to 6 days' incubation at 25° C. Cultures were made in flat medicine bottles; spores were removed by adding a little sterile water to the bottle and rubbing the agar surface gently with a glass rod.

PRELIMINARY EXPERIMENTS

Cultures on 30 ml. lots of medium in 100 ml. pyrex flasks were set up on five standard media (Weindling, Czapek-Dox, Raulin-Thom, Cornsteep and Peptone-Lemco-glucose). The composition of these media has been given elsewhere (Brian, Curtis & Hemming 1946). A crude grade of glucose was used in all these media. Results of periodical assays of fungistatic and antibacterial activity are shown in table 1. The data given show the greatest dilutions inhibiting germination of *Botrytis allii* conidia (i.e. B.A. units/ml.), growth of *Staphylococcus aureus* (i.e. *Staph.* units/ml.) or *Salmonella typhi* (i.e. *Salmonella* units/ml.).

Growth was best on Czapek-Dox, less vigorous on Weindling and Raulin-Thom, but very poor on Cornsteep and Peptone-Lemco-glucose. The assay results show that *Metarrhizium glutinosum* produces markedly fungistatic culture filtrates, particularly on Raulin-Thom, but that little, if any, antibacterial activity develops.

The superiority of Raulin-Thom over Czapek-Dox is also shown in table 2, where results are given for these two media adjusted to various pH values. The media were adjusted with caustic soda or hydrochloric acid to pH values of 3·0, 4·0, 5·0, 6·0 and 7·0 before autoclaving, but the pH changed considerably during autoclaving so that a range of pH 2·8 to 5·6 only was obtained. Crude glucose was again used in these media. Growth was noticeably inferior on media with initial pH below 4·0, and these same media also showed a rather slower development of fungistatic activity.

TABLE 1. FUNGISTATIC AND ANTIBACTERIAL ACTIVITY OF CULTURE FILTRATES FROM *METARRHIZIUM GLUTINOSUM* ON FIVE STANDARD MEDIA

activity	days growth	Weindling	Raulin-Thom	Czapek-Dox	Cornsteep	Peptone-Lemco-glucose
B.A. units/ml.	6	8	16	8	16	4
	8	32	256	32	—	4
	10	16	48	16	—	4
	12	16	32	16	—	4
Staph. units/ml.	6	—	2	- -	2	—
	8	—	—	- -	- -	—
	10	—	2	—	—	—
	12	2	4	—	—	—
Salmonella units/ml.	6	—	2	- -	2	—
	8	—	—	—	—	—
	10	—	2	—	—	—
	12	4	4	—	—	—

TABLE 2. FUNGISTATIC ACTIVITY (B.A. UNITS/ML.) OF CULTURE FILTRATE FROM *METARRHIZIUM GLUTINOSUM* ON CZAPEK-DOX AND RAULIN-THOM MEDIA INITIALLY ADJUSTED TO VARIOUS pH VALUES

medium	initial pH	days growth at 25° C			
		4	6	8	11
Czapek-Dox	2 8	—	4	32	16
	3·5	8	16	16	32
	3·8	6	12	32	32
	4 8	16	32	32	48
	5·5	4	16	32	16
Raulin-Thom	2·8	4	4	64	256
	3·6	4	4	128	128
	4·3	8	16	32	256
	4·9	8	32	64	256
	5·6	4	16	32	128

On the basis of these promising results experimental work was undertaken
(a) to investigate the possibility of securing a more favourable medium than
Raulin-Thom and (b) to develop methods of extraction of the active fungistatic
substance. These two lines of research were necessarily carried on simultaneously
but are dealt with separately below.

GROWTH-FACTOR REQUIREMENTS

Certain anomalies in experimental results suggested that crude glucose (used in
large batches for production purposes) was superior to pure dextrose for develop-
ment of fungistatic activity in culture filtrates. Brian (1946) has shown that this
same impure grade of glucose was superior to pure dextrose as a constituent of
media for production of gliotoxin by *Penicillium terlikowskii*. This superiority in
cultures of *Metarrhizium glutinosum* was confirmed by experiment (table 3);
production of active culture filtrates was more rapid, and higher maximum assays
were observed in both Raulin-Thom and Czapek-Dox media made up with crude
glucose. More noticeable than the effects on activity of culture filtrates were the
effects on growth and sporulation. Raulin-Thom medium produced largely sub-
merged mycelium; Czapek-Dox produced stout superficial felts sporulating very
sparsely with pure dextrose but heavily with crude glucose.

TABLE 3. COMPARISON OF CRUDE GLUCOSE AND PURE DEXTROSE AS CONSTITUENTS
OF CULTURE MEDIA FOR *METARRHIZIUM GLUTINOSUM*

days growth at 25° C	fungistatic activity (B A. units/ml.)			
	Czapek-Dox		Raulin-Thom	
	pure dextrose	crude glucose	pure dextrose	crude glucose
3	—	16	4	16
5	32	48	8	64
10	64	128	32	128
12	64	128	64	256
16	64	128	64	128

As a complete minor element mixture was added to all media it was considered
that the impurity in crude glucose producing the effects on sporulation and activity
of culture filtrates was most probably a vitamin or other growth factor. Accordingly
supplements of the following substances, in the concentrations listed, were tested:

Difco yeast extract	0·01 to 0·1 %	p-aminobenzoic acid	0·01 %
Peptone	0·01 to 0·1 %	l-glutamic acid	0·01 %
Marmite	0·01 to 0·1 %	aneurin hydrochloride	1 μg./ml.
dl-alanine	0·01 %	nicotinic acid	1 μg./ml.
glycine	0·01 %	riboflavin	1 μg./ml.
l-tryptophane	0 01 %	pyridoxin	1 μg /ml.
β-phenylalanine	0·01 %	biotin	0·1 to 1·0 μg./ml.
l-tyrosine	0·01 %		

Of these, yeast extract, peptone, marmite, aneurin and biotin produced marked stimulation of sporulation and small increases in fungistatic activity. *M. glutinosum* thus appears to be partially heterotrophic for aneurin and biotin. For practical purposes, optimum concentrations of these growth substances were best introduced into the medium by use of crude glucose, and this was always done for production of glutinosin.

THE SIGNIFICANCE OF THE FORM OF NITROGEN SUPPLIED IN CULTURE MEDIA

The form in which nitrogen is supplied has been shown by previous investigations to have a profound effect on the growth of fungi and on the course of metabolism. Accordingly, an experiment was set up in which six different nitrogen sources were compared   For this purpose a basal medium of the following composition was used

dextrose (A.R.)	50 0 g.	minor element concentrate	1·0 ml
potassium dihydrogen phosphate	1 0 g	distilled water	1000·0 ml
magnesium sulphate ($MgSO_4.7H_2O$)	0·5 g.		

The minor element concentrate had the following composition·

ferrous sulphate ($FeSO_4.7H_2O$)	0·1 g.	manganese sulphate ($MnSO_4$ $7H_2O$)	0·01 g.
cupric sulphate ($CuSO_4$ $5H_2O$)	0 015 g.	potassium molybdate	0 01 g.
zinc sulphate ($ZnSO_4$ $7H_2O$)	0·1 g.	distilled water	100·0 ml.

and was acidified with sufficient hydrochloric acid to prevent the formation of any precipitate To this basal medium various nitrogen compounds were added to give equal concentrations of nitrogen in each case, as follows

medium N · 2·3 g./l. potassium nitrate	*medium CN* . 2 0 g./l. calcium nitrate
medium AN 0·95 g./l. ammonium nitrate	*medium T* : 2·5 g /l. peptone
medium A · 2 2 g./l. ammonium tartrate	(Bacto-Tryptone)
medium AS · 1·6 g./l. ammonium sulphate	

The media were dispensed in 500 ml. lots in glass culture vessels. All media were adjusted to pH 5·0 before autoclaving. The results of periodical assays, pH measurements (glass electrode) and final dry weight of mycelium are shown in table 4.

The main conclusions to be drawn from these results are:

(1) Media containing nitrogen as potassium or calcium nitrate (media *N* and *CN*) are superior to all others for production of fungus mycelium. On these media heavy, much-folded, confluent felts, with moderate sporulation, were produced. These were followed in effectiveness by medium *T*, containing peptone nitrogen; on this medium a non-sporulating, folded, confluent felt was produced. The media containing nitrogen as ammonium sulphate or ammonium nitrate (media *AS* and *AN*) produced very little mycelium; a superficial felt was not formed and most of the mycelium was submerged and fragmented. It is of interest to note that the medium containing nitrogen as ammonium nitrate behaved as an ammonium medium rather than as a nitrate medium. Medium *A*, containing nitrogen as ammonium tartrate, produced quite a heavy crop of mycelium in the form of

a non-sporulating, little-folded, superficial felt. There is, therefore, a clear distinction between the media containing ammonium sulphate or ammonium nitrate and that containing ammonium tartrate.

TABLE 4. DEVELOPMENT OF FUNGISTATIC ACTIVITY, DRIFT OF pH AND DRY WEIGHT OF MYCELIUM PRODUCED IN CULTURES OF *METARRHIZIUM GLUTINOSUM* ON MEDIA WITH DIFFERING NITROGEN SOURCES

days growth	N	AN	A	AS	CN	T
			medium			
			fungistatic activity (B.A. units/ml.)			
5	12	2	24	6	2	96
8	64	16	1024	16	12	768
11	64	32	1024	16	32	768
14	32	16	512	8	64	512
19	16	32	512	12	32	512
			drift of pH			
0	5·0	5·0	5·4	5 0	4·8	5·2
5	6·4	4·1	4·3	3 5	5 4	3·5
8	6·8	4·0	3·4	3·0	6·1	3·0
11	6·7	4·1	3·2	3 3	6 2	3 3
14	6·0	3·8	3·2	3·2	5-5	3·2
19	6·4	3·5	3·4	3·0	6·1	3·0
			final dry weight (g) of mycelium per culture vessel			
19	4·91	0·49	2·17	0·57	4·03	1·47

(ii) There is no consistent correlation between production of fungus mycelium and development of fungistatic activity in the culture filtrates. The nitrate media (N and CN) producing heavy mycelial felts and the inorganic ammonium salt media (AS and AN) producing little mycelium were all relatively ineffective for production of fungistatic culture filtrates. The peptone medium (T) and the ammonium tartrate medium (A) were outstandingly good in this respect. The difference between the ammonium tartrate medium and the other ammonia media is thus again very striking.

(iii) In nitrate media (N and CN) the culture filtrates steadily rise in pH whereas the ammonia and tryptone media show a more gradual downward trend. Again, the ammonium nitrate medium behaves like the other ammonia media rather than like the nitrate media. There is no correlation between the pH changes and development of fungistatic activity.

STIMULATING EFFECTS OF ORGANIC ACIDS IN AMMONIA-NITROGEN MEDIA

The superiority of ammonium tartrate over ammonium sulphate as a nitrogen-containing constituent of media for development of fungistatic activity in cultures of *M. glutinosum* suggested that the tartrate radical must possess some special

value. Accordingly, the effect of additions to an ammonium sulphate medium (AS) of tartaric acid and subsequently of other organic acids was investigated. The acids were added in concentrations of 0·05 to 1 0%, and the media then adjusted to pH 4·0 with caustic potash before autoclaving. All experiments were carried out on 250 ml. lots of medium in 'Glaxo' vessels. The organic acids used can be conveniently grouped as follows:

(a) Dicarboxylic aliphatic acids, (b) tricarboxylic aliphatic acids, (c) monocarboxylic aliphatic acids, (d) amino-acids, (e) cyclic acids. These groups are discussed in turn below.

(a) *Dicarboxylic aliphatic acids*

The results with tartaric ($COOH.CHOH.CHOH COOH$), malic

$$(COOH.CHOH.CH_2.COOH),$$

succinic ($COOH.CH_2.CH_2.COOH$), malonic ($COOH.CH_2 COOH$) and oxalic ($COOH.COOH$) acids are summarized in tables 5 and 6 Each of these acids caused considerable changes in the course of metabolism, the main effects being (i) an increase in the dry weight of mycelium formed, (ii) an increase in the fungistatic activity of culture filtrates and (iii) an alteration in the pH drift of the medium.

The increase in dry weight of mycelium was accompanied by a change in the growth form. On the unsupplemented medium growth was sparse, submerged and particulate, in fact, was very similar in appearance to that obtained with most fungi in shaken cultures Addition of the organic acids led to formation of a surface mycelium, a strong, confluent, superficial felt being formed with the higher concentrations of acid. The increases in amount of mycelium formed were in some cases (e g. malonic acid) very large, but in all cases were clearly significant. The maximum increase in dry weight was in some cases obtained with less than 1 % of added acid, but where this was so, addition of further acid up to 1 % did not lead to a decrease in dry weight of mycelium formed.

The increases in fungistatic activity of culture filtrates were also very marked. There was evidence in some cases that the optimum concentration of added acid was below 1 % For example, in the cases of oxalic and succinic acids the addition of 1 % acid produced results definitely inferior to certain lower concentrations. Thus the increased fungistatic activity of culture filtrates could not be directly correlated with increased amount of mycelium produced.

The culture filtrates from the unsupplemented medium showed a characteristic downward drift of pH. Addition of these dicarboxylic acids slowed down or prevented this fall in pH or, in the case of the higher concentrations of malic and oxalic acids, substituted for it a rapid rise in pH. This effect was possibly in part due to a buffering effect, but in the cases where the pH rose rapidly, must have been due to an accumulation of hydroxyl ions resulting from assimilation of acid anions. The extent of the rise in pH can therefore be considered to be an indirect measure of the availability of the acids for purposes of assimilation.

TABLE 5. EFFECT OF ADDITION OF DICARBOXYLIC ACIDS TO MEDIUM AS ON FUNGISTATIC ACTIVITY AND pH OF CULTURE FILTRATES FROM *METARRHIZIUM GLUTINOSUM*

organic acid supplement	days growth	acid supplement %					acid supplement %				
		0	0.1	0.25	0.5	1.0	0	0.1	0.25	0.5	1.0
		B.A. units/ml.					pH				
d-tartaric	4	16	16	16	16	32	3.1	3.6	3.7	3 7	3.8
	8	24	16	16	32	32	2 8	3.5	3.3	3 6	3.6
	12	16	32	24	32	128	2.7	3.0	3.3	3.5	3.6
	18	32	16	16	32	64	2.6	3.2	3.5	3.6	3.7
dl-malic	4	2	32	32	48	64	3.1	3.3	3 7	3.8	3.9
	8	8	32	32	32	128	2.9	3.1	3.5	4.1	5.4
	12	8	32	32	48	128	3.1	3 1	3.4	5 2	5.8
	17	16	32	32	64	64	3.1	3 0	3 3	4 0	6.6
succinic	4	2	4	4	4	4	4 0	3 9	4 0	4.2	4 2
	8	4	16	16	8	16	3.2	3.3	3 8	4 0	4.1
	12	4	16	16	64	16	2 8	3 0	3 2	3.7	4 0
	18	4	16	16	128	32	2.4	2 6	2.7	3 6	4.0
malonic	4	2	2	2	2	4	4 4	4 2	4 4	4 2	4 3
	7	2	8	16	16	16	3 4	3.6	4 1	4 0	4.3
	11	8	32	32	64	32	2.7	3 3	3 6	3.9	4.2
	18	16	32	512	256	256	3 2	2.7	4 5	4 7	4 6
oxalic	4	2	32	12	32	16	3.0	4.2	4.8	4.5	4 2
	9	8	64	32	24	16	3.0	2 8	6.1	6.0	8 6
	14	24	128	96	48	24	2.7	2.9	6 1	7 6	8 0
	18	24	64	48	32	12	2 8	2.9	6 7	7 5	8 3

TABLE 6. PERCENTAGE INCREASE IN DRY WEIGHT OF MYCELIUM PRODUCED BY ADDITION OF DICARBOXYLIC ACIDS TO CULTURES OF *METARRHIZIUM GLUTINOSUM* ON MEDIUM AS

organic acid supplement	days growth	acid supplement %			
		0.1	0.25	0 5	1.0
d-tartaric	18	25	18	42	196
dl-malic	21	73	238	380	342
succinic	22	43	66	65	63
malonic	21	71	458	860	805
oxalic	24	197	232	546	572

(b) Tricarboxylic aliphatic acid

Results with citric acid ($COOH\ CH_2.C(OH)COOH.CH_2\ COOH$) are presented in table 7 Citric acid produced increases in fungistatic activity of culture filtrates and in weight of mycelium produced and, in the higher concentrations, arrested the fall in pH of the medium. Its effects were therefore in all ways similar to those of the dicarboxylic acids already mentioned.

TABLE 7. EFFECT OF ADDITION OF CITRIC ACID TO MEDIUM *AS* ON FUNGISTATIC
ACTIVITY AND pH OF CULTURE FILTRATES AND ON DRY WEIGHT OF MYCELIUM
PRODUCED IN CULTURES OF *METARRHIZIUM GLUTINOSUM*

days growth	acid supplement %					acid supplement %				
	0	0·1	0·25	0·5	1·0	0	0·1	0·25	0·5	1·0
		B.A. units/ml.						pH		
4	16	32	32	16	64	2·8	3·2	3·3	3 5	3·5
10	16	24	32	32	46	2 7	3 0	3 3	3 6	3 6
13	24	32	32	32	256	2 7	2 9	3 1	3 4	3 5
16	16	32	64	32	256	2·3	2·6	2·9	3·2	3·5

	acid supplement %			
	0 1	0·25	0·5	1 0
increase % in dry weight of mycelium (16 days' growth)	−2	19	33	112

(c) *Monocarboxylic aliphatic acids*

The results with acetic ($CH_3.COOH$), propionic ($CH_3.CH_2.COOH$), glycollic ($CH_2OH.COOH$) and pyruvic ($CH_3.CO.COOH$) acids are presented in tables 8 and 9. In preliminary experiments, the monocarboxylic acids were found to be toxic. Other investigators (e.g. Hoffman, Schweitzer & Dalby 1941) have shown that it is the undissociated molecule which is mainly responsible for toxicity. Accordingly, in addition to the experiments at pH 4·0, which gave a direct comparison with the experiments with dicarboxylic acids, the experiments were repeated (except in the case of pyruvic acid) at pH 6 5, under which conditions the acids would be almost completely dissociated.

None of the concentrations of acetic acid tested allowed growth in the medium of initial pH 4·0. At pH 6·5 the fungistatic activity of culture filtrates was greatly increased by 0·1 % acetic acid, and the weight of mycelium formed was increased progressively as the concentration of added acetic acid was increased. The pH effects were similar to those observed with some of the dicarboxylic acids.

Propionic acid did not lead to increase in fungistatic activity of culture filtrates. This was probably associated with the fact that even at pH 6·5 it greatly reduced the vigour of growth. It is worthy of note that, though it decreased the weight of mycelium produced at pH 6·5, its addition to medium *AS* did result in changing the form of growth from the submerged type to the surface type, though only islands of growth were produced which did not coalesce to form a confluent felt.

Glycollic acid was not toxic and, both at pH 4·0 and 6·5, led to an increase in fungistatic activity of culture filtrates, an increase in dry weight of mycelium formed and formation of a confluent surface felt of mycelium. Thus in all respects it behaved like the dicarboxylic acids.

The result with pyruvic acid was of particular interest. A concentration of 1·0 % was highly toxic (at pH 3·5) and no growth took place. At 0·5 % it was still

TABLE 8. EFFECT OF ADDITION OF MONOCARBOXYLIC ACIDS TO MEDIUM *AS* ON
FUNGISTATIC ACTIVITY AND pH OF CULTURE FILTRATES FROM *METARRHIZIUM
GLUTINOSUM*

organic acid supplement	days growth	acid supplement %							acid supplement %					
		0	0·05	0·1	0·25	0·5	1·0		0	0·05	0·1	0·25	0·5	1·0
		B.A. units/ml.							pH					
								initial pH 4·0						
acetic	4	4	no growth						3 6	no growth				
	9	8	no growth						2·9	no growth				
	14	12	no growth						2 7	no growth				
	17	8	no growth						2·6	no growth				
propionic	4	16	no growth						3·0	no growth				
	7	16	no growth						3 3	no growth				
	14	16	no growth						2 6	no growth				
	17	16	no growth						2·4	no growth				
glycollic	4	4	12	16	24	24	24		3 4	3 6	3 6	3 7	3 7	3 9
	9	4	12	12	16	32	128		3·1	3·1	3 4	3 5	3 6	3·9
	13	8	16	12	16	32	96		2 6	2·8	2·9	3·5	3·5	3·9
	17	8	16	16	24	32	64		2 5	2 5	2 7	3 0	3·5	3·9
pyruvic	4	4	8	8	16	—	—		3·3	3·3	3·9	4 2	4·5	4 2
	8	8	12	12	96	4	—		3 2	3·3	3·9	4 1	4·5	4 2
	12	16	24	24	64	96	—		3 2	3 1	3·7	4·1	4·4	4·2
	16	32	64	64	128	256	—		3 1	3·1	3·8	4·1	5·5	4·2
								initial pH 6 5						
acetic	4	8	—	—	—	—	—		5 0	5 8	5 5	5 6	6·2	—
	9	64	48	256	48	6	—		2·8	3 5	5·0	6·2	6·6	—
	14	64	64	256	32	16	—		2·5	3·0	5 2	7·2	8·0	—
	17	64	64	256	32	16	—		2 5	2·9	5·4	7·3	7·9	—
propionic	4	32	16	4	—	—	—		2·7	4 5	4 9	5·6	6 0	—
	7	64	16	4	2	—	—		2·5	4·5	4 8	5·3	5 9	—
	14	48	32	4	—	—	—		2·6	4·0	4 5	5·2	5·5	—
	17	48	16	6	2	—	—		2 4	4·2	4 4	5·0	5 5	—
glycollic	4	32	128	64	64	48	16		3 0	3 5	3 6	3 9	4 5	5·3
	9	64	96	32	128	256	128		3·0	3 3	3 2	3 6	4·4	4·7
	13	48	48	32	96	256	128		3·1	3 1	3 0	3·8	7·2	6·3
	17	32	48	32	64	64	64		2·7	2·8	2·8	3·8	8·0	7·8

TABLE 9. PERCENTAGE INCREASE IN DRY WEIGHT OF MYCELIUM PRODUCED BY
ADDITION OF MONOCARBOXYLIC ACIDS TO CULTURES OF *METARRHIZIUM
GLUTINOSUM* ON MEDIUM *AS*

organic acid supplement	days growth	acid supplement %					acid supplement %				
		0·05	0·1	0·25	0·5	1·0	0·05	0·1	0·25	0·5	1 0
		initial pH 4·0					initial pH 6·5				
acetic	21	no growth					65	205	280	300	—
propionic	20	no growth					−33	−58	−67	−78	—
glycollic	20	117	183	217	234	256	71	116	242	96	131
pyruvic	20	−13	28	91	281	−86					

toxic; growth was initially very slow, and after 8 days only very slight growth was observed. Growth then became very rapid, a thick superficial felt was formed and the activity of the culture filtrate rapidly surpassed that of all other media. At 0·25 % pyruvic acid and below that concentration, though the activity of the culture filtrate was superior to that of unsupplemented medium *AS*, all mycelium was of the submerged type.

TABLE 10. EFFECT OF ADDITION OF AMINO-ACIDS TO MEDIUM *AS* ON FUNGISTATIC ACTIVITY AND pH OF CULTURE FILTRATES FROM *METARRHIZIUM GLUTINOSUM*

organic acid supplement	days growth	acid supplement %					acid supplement %				
		0	0·1	0·25	0·5	1·0	0	0 1	0 25	0·5	1·0
				B.A. units/ml.					pH		
glycine	4	2	4	4	2	4	3 0	4·2	4·5	4 5	4 5
	8	6	8	32	96	48	2 9	3·2	3 3	3 5	6·0
	12	8	16	16	32	32	3·2	3·4	3·6	3·8	6 3
	16	16	24	48	64	64	3·2	3 4	3·6	4 2	7 1
dl-alanine	4	2	8	4	2	—	3 3	3·5	3·7	3·8	—
	10	16	16	12	16	—	2·9	3·1	3·2	3 4	—
	14	16	16	16	16	—	2·9	3·0	3·0	3 3	—
	18	32	32	16	32	—	2·7	3 0	3 0	3 3	—
dl-aspartic	4	—	32	32	64	32	3 6	3·4	4·5	4 3	4·5
	8	2	128	256	256	128	2 8	3·3	3·2	4 2	8·6
	12	—	64	256	256	64	2 3	3·1	3 0	6 7	8 5
	20	2	512	512	512	128	2·2	2·5	6 9	8·6	9·0
l-glutamic	4	32	32	32	32	48	2 8	3·0	3 6	3 7	3·9
	9	16	16	32	32	128	2 7	3 0	3 6	3·8	8·0
	13	24	16	48	48	96	2·7	2·9	3 6	3·6	8·5
	16	16	16	16	64	64	128	2 3	2 5	3·3	3·3	8 6

TABLE 11. PERCENTAGE INCREASE IN DRY WEIGHT OF MYCELIUM PRODUCED BY ADDITION OF AMINO-ACIDS TO CULTURES OF *METARRHIZIUM GLUTINOSUM* ON MEDIUM *AS*

amino-acid supplement	days growth	acid supplement %			
		0 1	0 25	0 5	1·0
glycine	20	6	70	301	245
dl-alanine	22	2	− 5	4	− 7
dl-aspartic	24	276	436	379	595
l-glutamic	16	30	43	142	111

It is of interest here to compare the results with the unsupplemented medium *AS* at pH 4·0 and 6·5. At the high initial pH more mycelium was formed (the average increase for the three experiments was 25 %) and rather higher assays were obtained, but growth in both cases was of the submerged type

(d) *Amino-acids*

Results with glycine ($CH_2.NH_2.COOH$), *dl*-alanine ($CH_3.CHNH_2.COOH$), *dl*-aspartic acid ($COOH.CH_2.CHNH_2.COOH$) and *l*-glutamic acid

$$(COOH.CH_2.CH_2.CHNH_2.COOH)$$
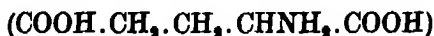
are given in tables 10 and 11. All except alanine produced increases in activity and increased weights of mycelium. The effects produced by glycine and glutamic acid were relatively small; aspartic acid was by far the most effective. The failure of alanine to stimulate is surprising, since the corresponding keto-acid—pyruvic acid—was quite effective.

(e) *Cyclic acids*

Benzoic and salicylic acids were tried as supplements to the medium *AS* but were found to be too toxic, either completely preventing growth or reducing it to negligible proportions whether the initial pH was adjusted to 4·0 or 6·5. A heterocyclic dicarboxylic acid, meconic acid, related in structure to the mould metabolic product kojic acid, was not toxic to *M. glutinosum*, but addition to medium *AS* was completely without effect on the course of metabolism.

Discussion

Considering the results so far it appears that addition of various aliphatic acids, monocarboxylic, dicarboxylic or tricarboxylic, to medium *AS* results in greatly enhanced growth of *M. glutinosum*, alters the growth form and greatly increases the fungistatic activity of culture filtrates. Acids of chain length from 2 to 5 carbon atoms have been found to be effective, though there appears to be a general tendency for those of shorter chain lengths to be most effective. In the development of a medium for mass production of glutinosin, choice would fall on one of the dicarboxylic acids, since these are less liable to be toxic at low pH than the monocarboxylic acids and less costly than the amino-acids. Further experimental work concerning the mechanism of the stimulatory action of these organic acids is described in the next sections.

RELATION BETWEEN ORGANIC ACIDS AND CARBON SOURCE IN AMMONIA-NITROGEN MEDIA

In table 12 results are given of an experiment in which medium *AS* was made up with a variety of alternative carbon sources (5 %) with and without a 0·5 % malic acid supplement. The carbon sources used included polysaccharides, disaccharides, monosaccharides and glycerol. It will be seen that none of the carbon sources gave good results alone, and that addition of malic acid increased fungistatic activity in each case. Without malic acid all growth was of the sparse, submerged type, whereas good superficial felts were formed with malic acid. Starch, dextrins and lactose in combination with malic acid were inferior to other carbon sources for production of fungistatic material, and growth on all lactose media was noticeably poor.

TABLE 12. COMPARISON OF VARIOUS CARBON SOURCES, WITH AND WITHOUT MALIC
ACID SUPPLEMENT, IN AN AMMONIUM SULPHATE MEDIUM, ON FUNGISTATIC
ACTIVITY AND pH IN CULTURES OF *METARRHIZIUM GLUTINOSUM*

carbon source	days growth at 25° C				days growth at 25° C			
	4	8	13	17	4	8	13	17
	B.A. units/ml.				pH			
dextrose	16	8	16	16	3 3	3·2	3·1	3·0
dextrose + malic acid	128	256	192	256	4·9	6·2	6·1	6 0
maltose	24	32	32	24	3·2	3·1	3·1	3·0
maltose + malic acid	256	1024	512	128	5·9	6 5	6 5	6 4
sucrose	32	24	32	32	3·3	3 1	3 1	3 0
sucrose + malic acid	96	128	192	128	4·6	6 0	6 4	6 5
lactose	—	—	—	—	3·7	3 4	3·4	3·1
lactose + malic acid	16	16	24	8	5·5	6·7	6 3	6·3
dextrin	—	—	2	2	3·5	3 4	3 2	3 2
dextrin + malic acid	128	128	128	128	4·9	6 5	6·8	6·7
starch	2	2	8	16	3 1	3·1	3 1	3·0
starch + malic acid	32	32	32	64	5 0	6 2	6 5	6·7
glycerol	16	24	32	32	3 2	3 0	3·1	3 0
glycerol + malic acid	256	256	128	128	4·5	4 9	6 8	6·7

THE MECHANISM OF STIMULATION BY ORGANIC ACIDS

In cases described in the previous sections maximum fungistatic activity has usually been observed under conditions of maximum growth. There were occasional exceptions; with oxalic acid, for instance, maximum fungistatic activity was achieved by addition of 0·1 % oxalic acid, whereas maximum growth was obtained with 0·5 or 1 % oxalic acid. In general, however, we shall be justified in treating the stimulatory effect of organic acids on growth and on development of fungistatic activity in culture filtrates as different aspects of the same basic phenomenon.

Four possible explanations of the stimulatory effect of these organic acids may be suggested·

(i) that the organic acids are superior to sugars, polysaccharides or glycerol as carbon sources, or that there is a deficiency of carbon source in the 5 % glucose medium which their addition removes;

(ii) that the fungus needs for purposes of metabolism a short carbon chain, in which case other substances than acids should be suitable;

(iii) that their effect on pH drift is responsible;

(iv) that the organic acids play an integral part in nitrogen metabolism.

These possibilities are considered in turn below.

Comparison with glucose as carbon source

Cultures of *M. glutinosum* were set up in 100 ml. flasks on medium *AS* salts, with malic acid or glucose as carbon source, or with combinations of the two, all being adjusted to pH 4·0. Results of assays and pH determinations are given in table 13.

Growth without a carbon source was negligible. With malic acid a very thin sporulating surface felt was formed, and with glucose the usual non-sporulating submerged mycelium. The combination of malic acid and glucose, on the other hand, supported vigorous surface growth. The fungistatic activity of culture filtrates followed the amount of growth closely. Thus it is the *combination* of glucose and organic acid as carbon source which is effective.

TABLE 13   EFFECT OF ADDITION OF MALIC ACID AND GLUCOSE AS CARBON SOURCES
TO SALTS OF MEDIUM *AS*, SEPARATELY OR IN COMBINATION, ON FUNGISTATIC
ACTIVITY AND pH IN CULTURES OF *METARRHIZIUM GLUTINOSUM*

carbon source	days growth at 25° C				days growth at 25° C			
	4	7	12	16	4	7	12	16
	B.A. units/ml.				pH			
none	—	—	—	—	4·4	5 4	5 7	5·2
malic acid (0·5%)	—	4	—	—	3 3	7·2	8 6	8·7
malic acid (1·0%)	4	8	4	4	3·4	5·6	8 4	8·2
glucose (5 0%)	4	16	16	8	3·1	2 7	2 8	2·5
glucose (5·0%)+malic acid (0 5%)	128	256	128	64	4·4	6·7	7 4	7·4
glucose (5 0%)+malic acid (1·0%)	128	128	64	64	4·4	4·8	7 2	8·4

In another experiment in which glucose concentrations of 2·5 to 10 0% were used, alone and in combination with malic acid, it was found that alteration of the concentration of glucose had little effect either on fungistatic activity of culture filtrates or on growth form. It did have some effect on the weight of mycelium produced, 2·5% glucose being noticeably inferior in this respect to the higher concentrations. This experiment confirms the conclusion that it is the combination of organic acid and glucose (or other suitable carbon source) that is essential to the development of high fungistatic activity and vigorous growth

Comparison with other substances with a short carbon chain

Acetone ($CH_3.CO.CH_3$), ethyl alcohol ($CH_3.CH_2 OH$) and glycerol

($CH_2OH.CHOH.CH_2OH$)

have been tested as supplements to medium *AS* in concentrations between 0·05 and 1·0% Though none of these was toxic, none, on the other hand, caused any growth stimulation, change in growth form or increase in fungistatic activity. Of these substances we have definite evidence that glycerol is readily metabolized by *M. glutinosum* but unlike the acids with a similar number of carbon atoms (e g. malonic) it caused no stimulation under these conditions. It therefore appears most probable that it is carboxylic acids that cause this specific type of stimulation rather than any metabolizable compounds of short chain length. Similarly, esters are not effective and the carboxyl group must therefore be considered to be essential.

Effect on pH *drift*

All the organic acid supplements, at the higher concentrations at least, affected the pH drift. This took the form either of slowing down or preventing the normal fall in pH of medium *AS* or, in some cases, of substituting for the slow fall in pH a rapid rise to pH 8·0 or thereabouts. It might be supposed that these changed pH relationships might account for the better growth and higher fungistatic activity of culture filtrates. A close inspection of the data already presented will show that, whereas there is certainly a close correlation between a change in the pH drift, increases in amount of mycelium formed and increase in fungistatic activity of culture filtrates, the relation between these cannot be considered to be causal as each of these three factors tends to move to some extent independently. In table 4 comparison of medium *A* (ammonium tartrate) and medium *AS* (ammonium sulphate) shows that the presence of tartrate led to great increases in growth and fungistatic activity without change in pH drift. Similar results are obtained (table 5) with additions of 0 1 % malonic or oxalic acids to medium *AS*. A more likely explanation of the general relationship between pH, growth and development of fungistatic activity is that both growth and production of fungistatic material depend on utilization of the organic acid, and any large utilization of organic acid is bound to result in accumulation of excess hydroxyl ions and hence to an increase in pH. Assimilation of small quantities of acid would not affect the pH as this would be masked by the buffering action of the medium. Though this may explain the basic relations between these three processes it should not be supposed that there are not other interactions between pH and organic acid utilization. This is shown by the following experiment

Medium *AS* and *AS* with 1·0 % malic acid added were adjusted to initial pH 4 0, 5·0 and 6·5 with KOH and HCl. Results of assays, pH measurements and growth measurements are shown in table 14.

These results show a series of interesting relationships With increasing initial pH the weight of mycelium increases both in the series without malic acid and that with malic acid. The increases in weight produced by adjusting the initial pH to a high value in the series without malic acid are small compared with the effect of adding malic acid. Growth in all media without malic acid was of the submerged type.

Increasing initial pH in the series without malic acid led to small increases in fungistatic activity of culture filtrates, but in the series with malic acid fungistatic activity fell from a very high level in the medium with initial pH 4 0 to a level similar to that of the media without malic acid when the initial pH was raised to pH 6·5.

The pH drifts show points of interest, in the media with no malic acid the pH fell to much the same low level whatever the starting pH. Addition of malic acid to the medium at initial pH 4·0 led, as usual, to a rapid upward trend in pH. Where the medium was adjusted to pH 5·0, the upward trend was delayed, and with initial

pH 6·5 no great rise in pH took place. This experiment, with slight modifications, has been repeated three times with almost identical results.

There is obviously, therefore, a complicated series of relationships between nitrogen metabolism, presence of organic acids, pH, type and amount of growth and development of fungistatic activity, the explanation of which will require much more exact physiological investigation. Nevertheless, it may be concluded that, although pH relationships are important, the stimulating effects of malic and other organic acids on growth are not directly due to their effects on the pH drifts within the medium.

TABLE 14. EFFECT OF VARIATION OF INITIAL pH OF MEDIUM ON FUNGISTATIC ACTIVITY, pH OF CULTURE FILTRATES AND DRY WEIGHT OF MYCELIUM IN CULTURES OF *METARRHIZIUM GLUTINOSUM* ON MEDIUM *AS* WITH AND WITHOUT MALIC ACID SUPPLEMENT

medium	days growth	initial pH			initial pH		
		4·0	5·0	6·5	4·0	5·0	6·5
		B.A. units/ml.				pH	
AS	5	8	16	4	3·0	3·0	6·3
	8	8	16	16	3·0	3·4	5·3
	11	6	12	24	2·7	2·8	3 6
	15	12	16	32	2·4	2·5	2 7
	19	16	32	32	2·4	2 6	2·6
AS + 1·0 % malic acid	5	48	64	—	4·7	5·0	6·4
	8	128	64	4	5·9	5·1	6·1
	11	128	48	16	7·0	5 6	5·6
	15	128	128	16	7·2	6·4	5·6
	19	64	96	24	8 0	8·1	6·8
		dry weight of mycelium as % of medium *AS* at pH 4·0					
AS	22				100	176	169
AS + 1 0 % malic acid	22				295	352	700

Assimilation of ammonia by condensation with organic acids

There is abundant evidence (Chibnall 1939) that in the higher plants nitrogen metabolism and organic acid metabolism are closely interrelated. Further, the organic acid metabolism has been shown by Vickery, Puchner, Wakeman & Leavenworth (1940) to be greatly influenced by the form in which nitrogen is supplied, organic acids rapidly disappearing if nitrate is replaced by ammonia nitrogen. It appears that, in general, ammonium salts do not accumulate in plants, as they are toxic, and that absorption of ammonia ceases unless ammonia can be rapidly combined in other forms. The usual first stage in ammonia metabolism is believed to be condensation of ammonia with α-ketonic acids ($R.CO.COOH$) to form the corresponding α-amino-acids ($R.CHNH_2.COOH$). A special and important case of this type of reaction is the condensation of ammonia with the ketonic acids oxaloacetic acid ($COOH.CO.CH_2.COOH$) and α-ketoglutaric

acid (COOH.CO.CH$_2$.CH$_2$.COOH), forming the amino-acids aspartic acid (COOH.CHNH$_2$.CH$_2$.COOH) and glutamic acid

$$(\text{COOH.CHNH}_2.\text{CH}_2.\text{CH}_2.\text{COOH})$$

respectively. These two condensations have special significance in that by a process of transamination aspartic and glutamic acids can transfer their amino-groups to α-keto-acids, being themselves transformed back to the parent oxaloacetic and α-ketoglutaric acids. These two acids can therefore act as reservoirs of nitrogen, storing up ammonia as amino-nitrogen until it is required for amino-acid synthesis. This storage function is increased by the fact that if excess ammonia is present a further molecule of ammonia can be condensed with aspartic and glutamic acids forming respectively the amides asparagine (COOH CHNH$_2$.CH$_2$.CONH$_2$) and glutamine (COOH.CHNH$_2$.CH$_2$.CH$_2$.CONH$_2$). In the higher plants, then, under normal conditions ammonia-nitrogen can be absorbed as long as the necessary α-ketonic acids, especially oxaloacetic and α-ketoglutaric acids, are present. These are normally produced from breakdown of carbohydrate. In the case of plants with very low carbohydrate reserves, in the dark, when no photosynthesis can take place, ammonia cannot be assimilated unless a carbohydrate (e.g. glucose) is supplied (Prianischnikow 1922; Smirnov 1923). Plants supplied with nitrate-nitrogen can accumulate nitrogen as nitrate within the cell, the highly probable eventual reduction to ammonia, before synthesis of amino-acids, only taking place in small quantities as required.

This brief survey of ammonia absorption in higher plants is suggestive. There is every reason to suppose that ammonia absorption in the fungi follows a similar course of condensation with α-ketonic acids produced by carbohydrate breakdown. In fungi, as in most living organisms, considerable possibilities exist for the inter-conversion of 3-, 4- and 5-carbon acids (Krebs cycle) and, in the case of fungi, possibilities for formation of these from 2-carbon acids are usually present (*Aspergillus niger*, for example, can produce citric acid from such 2-carbon acids as acetic and glycollic acids). In most fungi such acids are usually produced, sometimes in great quantity, and from these, by the transformations of the Krebs cycle in the cell, α-ketoglutaric and oxaloacetic acids could be formed. If we assume that *Metarrhizium glutinosum* is unusual in that it is unable (in the presence of ammonia-nitrogen) to carry out the process of splitting glucose or other carbohydrates into the necessary 3- or 4-carbon acids in sufficient quantity, then the stimulating effect of adding such acids to ammonia-nitrogen cultures is at once explained. Losses of physiological function of this type are now well known; of interest in this connexion is the loss by certain X-ray-induced mutants of *Neurospora crassa* of the capacity to synthesize certain amino-acids, unless the corresponding α-keto acids are supplied in the medium (Bonner, Tatum & Beadle 1943; Regnery 1944). In the case of *Metarrhizium glutinosum* it appears most likely that the block in the chain of breakdown from carbohydrate to the acids of the Krebs cycle is in the step triose-phosphate to pyruvic acid rather than in the step monosaccharide to triose-

phosphate. This is supported by the fact that glycerol-ammonia media also require the addition of 3-, 4- or 5-carbon acids for adequate growth of the fungus.

A number of other observations can be accounted for by this hypothesis. It has been found in several experiments, not reported in detail here, that addition of malic or other organic acids to nitrate-nitrogen media does not produce stimulation. Secondly, it has been found that asparagine, aspartic acid, glutamic acid and peptone are excellent nitrogen sources for growth and production of fungistatic culture filtrates, and such media are not appreciably, if at all, improved by the addition of malate. These facts are all in accord with the theory that the organic acids are specifically concerned with ammonia assimilation.

The main weakness of the hypothesis is the total lack of knowledge of the very different paths of metabolism that must be followed when nitrogen is supplied as nitrate. It is generally assumed that nitrate is reduced to nitrite and thence by several stages to ammonia, which is then condensed with α-ketonic acid in the manner already described, but in presence of nitrate-nitrogen apparently *M. glutinosum* can produce the necessary α-ketonic acids by carbohydrate cleavage. This is not entirely surprising, as it has several times been observed that different breakdown products from carbohydrates are produced if nitrate-nitrogen is replaced by ammonia-nitrogen, and, in this connexion, it is worth noting that Hoagland (1944) has suggested that the oxygen content of nitrate may be of significance in higher plants under conditions of poor aeration.

In *M. glutinosum* there appears to be a definite antagonism between the nitrate path of nitrogen metabolism and the ammonia path. With nitrate, growth is vigorous; with ammonia-nitrogen, in the absence of organic acids, growth is very poor; if nitrogen is supplied as ammonium nitrate growth is exactly as it would be with any inorganic ammonium salt. Further, if malic acid is added to a nitrate medium no effect can be seen; if added to an ammonium-nitrogen medium great growth stimulation results, if malic acid is added to an ammonium-nitrate medium the same growth stimulation is observed as with ammonium sulphate. In the presence of ammonia-nitrogen then, the nitrate path of metabolism, even when nitrate is present in considerable quantity, is closed.

Types of nitrogen metabolism of fungi

M. glutinosum is not unique in assimilating ammonia-nitrogen efficiently only in the presence of certain organic acids. Leonian & Lilly (1940), Burkholder & McVeigh (1940) and Bernhard & Albrecht (1947) have shown that *Phycomyces blakesleeanus* similarly cannot utilize inorganic ammonium salts, whereas ammonium salts of certain organic acids are as effective as asparagine as nitrogen sources. The effective acids for *P. blakesleeanus* are, in the main, the same as those effective for *Metarrhizium glutinosum*. These authors have suggested no explanation for the stimulating effect, though the data of Bernhard & Albrecht (1947) support our conclusion that it is not the effect of the organic acids on pH drift that is responsible for the growth stimulation. Leonian & Lilly (1940) recognize the synergic relation-

ship between the organic acids and ammonium salts, and regard the free carboxyl group, as we have done, to be essential. All the results of these authors can be accounted for by the hypothesis that in *Phycomyces blakesleeanus*, as in *Metarrhizium glutinosum*, the power of the fungus to produce 3-, 4- and 5-carbon acids is limited or absent, and that as a consequence assimilation of ammonia by condensation with α-keto-acids is correspondingly impeded. It should be noted that *Phycomyces* differs from *Metarrhizium* in that it is unable, under any circumstances, to utilize nitrate-nitrogen.

Fungi have been grouped physiologically on the basis of their nitrogen metabolism by Steinberg (1939) as follows:

(*a*) those capable of utilizing nitrate, ammonia and organic nitrogen;

(*b*) those capable of utilizing ammonia and organic nitrogen but not nitrate nitrogen;

(*c*) those capable of utilizing only organic nitrogen.

Similar classifications have been developed by Robbins (1937) and by Benecke & Jost (1924). These types have been considered to have developed by a process of physiological evolution as a result of progressive genetic losses of synthetic function (Knight 1938, Lwoff 1943) It is now obvious that this method of classification is not adequate. A distinction must be made between those fungi that can use inorganic ammonium salts and those that require the additional presence of organic acids. Further, this classification assumes that fungi which can utilize nitrate-nitrogen can also utilize inorganic ammonium salts, our experience with *M. glutinosum* has shown that this is not the case.

The common mould *Scopulariopsis brevicaulis* is another example of this physiological type. A comparative study of nitrogen metabolism of fungi must be undertaken, using a wide range of organisms, before any physiological classification can be confidently developed.

EXTRACTION AND PURIFICATION OF GLUTINOSIN

Methods of extraction

The results of various extraction experiments are shown in table 15. In preliminary experiments 100 ml quantities of culture filtrate were extracted by shaking with petrol ether (b.p. 40 to 60° C), ether, chloroform and *n*-butyl alcohol in separating funnels.

Of these four solvents all except chloroform were effective, but as the water-solubility of ether and *n*-butyl alcohol would lead to considerable losses of solvent, petrol ether was adopted initially as a method of extraction for large-scale culture. On evaporation of petrol ether extracts to dryness a pale yellow gum with embedded crystalline material was obtained. On dissolving this in a little hot ethyl alcohol a white, microcrystalline solid separated out on cooling; this substance had high antifungal activity and was named glutinosin. A brief chemical and physical

characterization of glutinosin has already been published (Brian & McGowan 1946), analyses and molecular weight determinations indicate that glutinosin has the molecular formula $C_{46}H_{60}O_{16}$.

TABLE 15. EXTRACTION OF GLUTINOSIN FROM CULTURE
FILTRATES BY ORGANIC SOLVENTS

solvent	quantity of solvent	activity extracted (as % of that originally present in culture filtrate)
petrol ether	3 times with $\frac{1}{10}$ vol.	94 (50 to 78*)
ether	3 times with $\frac{1}{10}$ vol.	94
ether	once with $\frac{1}{8}$ vol followed by once with $\frac{1}{10}$ vol.	98·5
ether	once with $\frac{1}{8}$ vol.	94
chloroform	3 times with $\frac{1}{10}$ vol.	50
n-butyl alcohol	3 times with $\frac{1}{10}$ vol.	98·4
cyclohexane	3 times with $\frac{1}{10}$ vol.	75
benzene	3 times with $\frac{1}{10}$ vol.	100

* These figures obtained in large-scale production batches.

On a large scale the petrol ether method of extraction did not show great efficiency; often as much as half the activity remained in the culture filtrate after extraction. As a result of this experience the possibility of extraction with cyclohexane or benzene was investigated. Extraction with cyclohexane was no better than with petrol ether but benzene was considerably better. On comparing the yields from the same culture filtrate, petrol ether gave 50 mg./l. glutinosin while benzene gave 70 mg./l.

Finally, charcoal treatment of culture filtrates was investigated. It had been found very early in the course of this work that treatment of culture filtrates with 10 g./l. of activated charcoal completely removed all activity. Little activity was recovered from the charcoal by elution with ether, and with ethanol, though all the activity could be recovered, the material was very impure and did not crystallize satisfactorily. It was eventually found that much better results were obtained by elution of the charcoal with hot benzene For this purpose a Soxhlet apparatus was originally used, but it was later found that the time of extraction could be reduced and better yields obtained by means of a Gallenkamp Universal Extractor. With this apparatus no thimble was used, but a plug of cotton-wool was placed in the base of the extractor and a slurry of charcoal in benzene poured on to the plug. Hot solvent percolates through the carbon back to the distilling flask continuously, 6 hr. extraction being found to be sufficient. The better results thus obtained are considered to be associated with a more even flow of solvent through the carbon as compared with that in a Soxhlet thimble where flow down the axis is probably slow. The carbon-benzene method of extraction gave higher yields of a more pure product than did direct extraction of the culture filtrate with benzene.

Yields

Table 16 gives, in round figures, the magnitude of yields obtained in batches of 10 to 15 l. of culture filtrate. These figures emphasize the superiority of malate or tartrate media and the charcoal method of extraction. For routine production of glutinosin the charcoal method of extraction is now used with an easily prepared culture medium of the following composition:

glucose (crude)	50 0 g.	magnesium sulphate	0·5 g.
phosphoric acid	0·75 g.	minor element concentrate	1·0 ml
malic acid	10 0 g.		

These constituents are dissolved in rather less than 1 l. of water, adjusted to pH 4·0 with a 50/50 mixture of 5N-potash and 5N-ammonia and then made up to 1 l. With this medium, yields of glutinosin of the order of 100 mg /l. can be obtained.

TABLE 16. YIELDS OF GLUTINOSIN (ONCE CRYSTALLIZED FROM ETHYL ALCOHOL)

medium	method of extraction	yield (mg./l.)
Czapek-Dox (2 5% glucose)	petrol ether	5 to 15
Raulin-Thom (2·5% glucose)	petrol ether	7 5 to 20
Raulin-Thom (2·5% glucose) + 0·25% malic acid	petrol ether	40 to 50
Raulin-Thom (2 5% glucose) + 0·25% malic acid	benzene	70
Raulin-Thom (5·0% glucose) + 0·25% malic acid	benzene	75 to 120
medium *T*	benzene	25
medium *T*	carbon	60
medium *A*	benzene	70
medium *A*	carbon	100
medium *AS*	carbon	5
medium *AS* + 1% malic acid	carbon	100

BIOLOGICAL ACTIVITY OF GLUTINOSIN

Stability of aqueous solutions

Before embarking on an investigation of the biological activity of glutinosin it was considered necessary to examine its stability in aqueous solution in relation to pH. Solutions of glutinosin (50 μg./ml.) were made up in McIlvaine's citric acid-phosphate buffers in a range from pH 3·1 to 7·8, stored at 25° C and assayed periodically by the standard *Botrytis allii* spore germination technique. Results are shown in table 17. The solutions of glutinosin are relatively stable, as fungistatic activity only declines slowly over a period of 3 weeks, unlike viridin (Brian *et al.* 1946) or gliotoxin (Brian & Hemming 1945) which lose their activity much more rapidly. Except for a suggestion that at pH 3·1 the decline in activity started less rapidly, there is little indication of any relation between pH and the rate of inactivation. When similar solutions (in this case 100 μg./ml) were autoclaved for 20 min. at 15 lb./sq. in. (see table 18) it became clear that the more acid solutions were most stable. Thus if glutinosin were included in the usual culture media before autoclaving we should expect a loss of about half of the activity during the process of sterilization.

TABLE 17. FUNGISTATIC ACTIVITY (B.A. UNITS/ML.) OF SOLUTIONS OF GLUTINOSIN IN MCÍLVAINE'S BUFFER STORED AT 25° C

days storage at 25° C	pH of solution					
	3·1	4 1	5·2	6·1	7·0	7·8
—	32	32	24	24	32	32
1	32	32	24	32	24	16
2	32	24	16	24	32	16
3	24	16	16	16	12	24
4	24	16	24	24	12	12
7	24	12	16	24	16	12
9	16	8	8	8	12	12
14	12	8	8	8	8	12
18	12	8	8	8	8	8
22	8	8	6	6	6	8

TABLE 18. EFFECT OF AUTOCLAVING SOLUTIONS OF GLUTINOSIN ON THEIR FUNGISTATIC ACTIVITY

	pH of solution					
	3·3	4 2	5 3	6 5	7 0	7·5
before autoclaving	128	128	128	128	96	128
after autoclaving (20 min. at 15 lb./sq.in.)	64	64	64	48	32	8

Toxicity to fungi

Data relating to the toxicity of glutinosin to fungi are presented in tables 19 and 20. In table 19 figures for toxicity in agar media are given; in this experiment glutinosin was included in Difco prune agar (pH 5·5) before autoclaving; the final concentration of glutinosin would then be one-half to one-quarter of that originally added if one takes into account loss due to autoclaving and to gradual loss in activity over the 7 days of the experiment All fungi grew vigorously on the prune agar without glutinosin except *Hydnum coralloides*, which showed little growth after 3 days though it was growing well after 7 days. In table 20 results of germination tests are given in Czapek-Dox medium (pH 4·8) and Weindling's medium (pH 3·5).

Results by the different test methods gave the same general order of susceptibility for the different fungi though the toxicity as judged by the spore-germination test appears greater than that judged by the agar test. Glutinosin was more toxic in Weindling's medium than in Czapek-Dox. The most noticeable feature of these results is the extreme specificity of glutinosin. Some fungi, as, for example, *Mucor mucedo*, *Byssochlamys fulva*, *Hydnum coralloides*, *Penicillium digitatum* and *Phoma betae*, were completely inhibited by 5 μg./ml. in agar, whereas under similar conditions such fungi as *Syncephalastrum racemosum*, *Gibberella saubinetti*, *Metarrhizium glutinosum* and *Trichoderma viride* were not at all inhibited by 100 μg./ml. The high resistance to glutinosin of *Fusarium graminearum* in spore-germination tests

previously published (Brian & McGowan 1946) has not been confirmed, though this fungus is moderately resistant in the agar tests reported here. The very great differences between the susceptibility of related species were most remarkable; examples are *Mucor mucedo* and *M. erectus* or *Penicillium digitatum* and *P. expansum*.

TABLE 19. TOXICITY OF GLUTINOSIN TO FUNGI IN PRUNE-AGAR CULTURES

fungus	3 days' incubation				7 days' incubation			
	100	25	5	1	100	25	5	1
	μg /ml. glutinosin				μg /ml. glutinosin			
PHYCOMYCETES								
Mucor erectus Bain	1	1	2	3	1	3	3	3
M. mucedo (+) Bref.	0	0	0	1	0	0	0	2
M. mucedo (−) Bref.	0	0	0	0	0	0	0	2
Phytophthora cryptogea Pethybridge	0	2	3	3	2	3	3	3
P. palmivora Butler	0	0	0	2	0	0	2	3
Syncephalastrum racemosum Schrect.	3	3	3	3	3	3	3	3
Thamnidium elegans Link	0	0	1	2	0	0	1	3
ASCOMYCETES								
Byssochlamys fulva Olliver & Smith	0	0	0	0	0	0	0	3
Chaetomium elatum Kunze & Schmidt	0	0	2	3	0	1	2	3
C. globosum Kunze	2	2	3	3	2	2	3	3
Endomycopsis albicans (Vuill.) Dekker	0	1	1	2	0	1	3	3
Gibberella saubinetii (Mont.) Sacc.	2	3	3	3	3	3	3	3
Melanospora pampeana Speg.	2	3	3	3	2	3	3	3
Neurospora crassa Shear & Dodge	0	0	0	0	0	0	2	3
Saccharomyces cerevisiae Hansen	0	0	1	2	0	0	3	3
Stereum purpureum Fr.	0	0	0	0	0	0	1	1
BASIDIOMYCETES								
Hydnum coralloides Scop.	0	0	0	0	0	0	0	0
FUNGI IMPERFECTI								
Aspergillus niger van Tiegh	1	3	3	3	2	3	3	3
A. oryzae (Ahlb.) Cohn	0	3	3	3	1	3	3	3
Botrytis allii Munn.	0	0	1	2	0	0	2	3
Fusarium caeruleum (Lib.) Sacc.	1	2	3	3	2	2	3	3
F. graminearum Schwabe	1	2	3	3	2	3	3	3
Metarrhizium glutinosum S. Pope	3	3	3	3	3	3	3	3
Monilia sitophila (Mont.) Sacc.	0	1	1	2	2	2	3	3
Penicillium digitatum Sacc.	0	0	0	1	0	0	0	1
P. expansum Link	0	2	2	2	2	2	2	3
P. gladioli McCull. & Thom.	0	1	1	2	0	2	3	3
Phoma betae Frank	0	0	0	0	0	0	0	2
Stachybotrys atra Corda	2	2	3	3	2	2	3	3
Torulopsis utilis (Henn.) Lodder	0	3	3	3	1	3	3	3
Trichoderma viride Pers. ex Fries	2	2	3	3	3	3	3	3
Trichothecium roseum Link	2	2	3	3	2	2	3	3
Verticillium cinnabarinum Reinke & Berth.	1	2	3	3	2	3	3	3
V. dahliae Kleb.	0	1	3	3	1	2	3	3

0 = no growth; 1 = trace of growth, 2 = marked reduction in growth; 3 = normal growth.

TABLE 20. TOXICITY OF GLUTINOSIN TO FUNGI IN SPORE GERMINATION TESTS
(LEAST INHIBITING CONCENTRATIONS IN μG./ML.)

fungus	Czapek-Dox		Weindling	
	complete inhibition	50% inhibition	complete inhibition	50% inhibition
PHYCOMYCETES				
Mucor mucedo (+) Bref.	1·6	0·8	—	—
Syncephalastrum racemosum Schrect.	12·5	6·25	—	—
Thamnidium elegans Link	0·4	0·2	—	—
FUNGI IMPERFECTI				
Aspergillus niger v. Tiegh.	25 0	12·5	6 25	3·1
Botrytis allii Munn.	0·8	0·4	0·2	0·1
Fusarium caeruleum (Lib.) Sacc.	3 1	1·6	0·8	0·2
F. graminearum Schwabe	3·1	1·6	3·1	1·6
Penicillium digitatum Sacc.	3·1	1 6	0·8	—
P. expansum Link	>50·0	25·0	25·0	6·25
P. gladioli McCull & Thom	3·1	0·4	0·8	0·2
Stachybotrys atra Corda	1 6	0·8	0·8	—
Stemphylium sp.	50·0	25 0	12·5	6·25
Trichoderma viride Pers. ex Fries	>50·0	>50·0	25·0	12·5
Trichothecium roseum Link	0·8	—	0·4	0·2
Verticillium cinnabarinum Reinke & Berth.	25·0	6·25	—	—

Toxicity to bacteria

Metarrhizium glutinosum culture filtrates (table 1) showed little, if any, anti-bacterial activity, and pure glutinosin has been found to be similarly inactive. The limit of solubility of glutinosin in nutrient broth is near 50 μg./ml., and at that concentration growth of the following organisms was not prevented: *Bacillus brevis*, *B. mycoides*, *B. mesentericus*, *B. subtilis*, *Escherichia coli*, *Micrococcus lysodeikticus*, *Salmonella typhi* and *Staphylococcus aureus* (two strains). *Bacillus lactis aerogenes* and one strain of *Staphylococcus aureus* were inhibited by 50 μg./ml. but not by 25 μg./ml. of glutinosin.

A DERMATITIC SUBSTANCE PRODUCED BY *METARRHIZIUM GLUTINOSUM*

After handling the first large batches of culture filtrates from this organism the writers and a number of assistants developed a severe facial inflammation. This developed some 24 to 48 hr. after handling the culture filtrate. The inflammation took the form of a purplish red rash with raised weals. The eyelids were very painfully affected, being inflamed and swollen. The inflammation died down after 3 or 4 days except on the eyelids, which remained very painful for several days longer. Finally, the skin on the eyelids, lips and most of the face peeled off. No after-effects were noticed.

Similar symptoms have been observed from time to time under conditions where there was no possibility of any culture filtrate coming into contact with the face;

the responsible agent is therefore volatile. By taking care to handle all culture filtrates in a well-ventilated room and by liberal use of barrier creams, it has been possible to avoid any further severe facial dermatitis.

On one occasion one of us (H.G.H.), after sucking up a culture filtrate in a pipette, developed a severe pharyngitis without any sign of actual infection. Another of us (P.W B.), after smelling a concentrate (see below), developed a painful nasal catarrh with slight haemorrhage.

It has been demonstrated that the inflammation is not an infection by the fungus. If small filter-paper disks are soaked in culture filtrate and strapped to the inner surface of the forearm, a severe inflammation, not unlike a third-degree burn, develops in 24 to 48 hr. The inflammation disappears after a week or so, but a pigmentation of the skin develops and persists for much longer. Similar effects are produced by ether, petrol ether, benzene or *n*-butyl alcohol extracts from the culture filtrate. Thus in the normal process of extraction of glutinosin the dermatitic substance is also extracted and concentrated. It has been established that pure glutinosin is not dermatitic.

It is therefore clearly established that *Metarrhizium glutinosum* produces, in the course of metabolism, a volatile dermatitic substance, soluble in water but more soluble in various organic solvents, and that this substance is distinct from glutinosin.

Only one other record of a substance with similar properties being produced by a fungus can be traced, and that is for the fungus *Stachybotrys alternans* Borod. by a number of Russian workers (Drobotko 1945, Moschani 1940, Salikov 1940; Vertinsky 1940) This fungus is associated with a disease of horses, known as stachybotryotoxicosis, caused by eating mouldy hay infected with the fungus The disease is characterized by catarrh with haemorrhage and ulceration of the mucosae of the mouth, nose and throat, followed, when the disease persists, by fever, staggering gait and death. At autopsy, a fatal case usually shows a general haemorrhagic gastro-enteritis with widespread haemorrhage in the lymphatic glands, spleen and other organs. It was shown that the disease could be produced by feeding animals on agar cultures of *St alternans*, and that ether extracts of the fungus or of culture media on which it had grown produced effects on human volunteers very similar to those produced by the *Metarrhizium* dermatitic product.

Cultures of several strains of *Stachybotrys atra* and *Memnoniella echinata*, considered by Bisby (1943) to be synonymous with *Stachybotrys alternans*, have failed in our experience to produce any dermatitic material.

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CORRIGENDA

p. 52, line 1.   For 'homolateral' read 'contralateral'.

p. 52, lines 3 and 4.   For 'reaching the ear from the contralateral carotid' read 'reaching this ear from the infused carotid'.

p. 87, line 6.   For 'is' read 'in'

p. 99, line 32.   For 'before this' read 'after this'

The Kodak Research Laboratories

By C. E. Kenneth Mees, F.R S.

[Plates 12 and 13]

In 1880 George Eastman commenced the manufacture and sale of gelatin photographic dry plates in Rochester, New York. From that undertaking, the Eastman Kodak Company, incorporated as an American company in 1902, has developed. In 1912 Mr Eastman decided to organize a laboratory, independent of the factory laboratories, which should carry out work on both the science and practice of photography. He was influenced by his observation of the success of industrial research under Dr Whitney's direction at the research laboratory of the General Electric Company in Schenectady, New York, U.S.A. and of the laboratories of the great German dye works He had been particularly impressed by the work done by the Bayer Company at Elberfeld.

In 1906 I had completed my thesis for the doctorate of science at University College, London, the subject being the theory of the photographic process, and had joined the old-established but very small firm of Wratten and Wainwright, Ltd , of Croydon as joint managing director. At Croydon both the conduct of research on photography and its application to the manufacture of photographic materials were continued actively, so that by 1912 many new materials had been introduced, especially panchromatic plates and the light filters and dark-room safe-lights required for their use, and the little firm was flourishing.

In January 1912 Mr Eastman invited me to go to Rochester, New York, to organize and direct the new laboratory. To enable me to do so, he purchased Wratten and Wainwright, Ltd , and transferred its production to the Kodak Works. At that time the Eastman Kodak Company had some 7000 employees in Rochester, of whom about half were in the film and paper factory of the Kodak Park Works. There were about 3000 employees elsewhere, including nearly 2000 in England. Besides Kodak Park, the Rochester factories included camera factories, while there were film, plate, and paper factories in Toronto, Melbourne and Harrow, and two small specialized factories in the United States.

At the present time the total employees of the Kodak companies number about 60,000, 44,000 of them being in the United States; of these 32,000 are in Rochester, including over 18,000 in the Kodak Park Works. The factories have been increased by a great chemical works at Kingsport, Tennessee, producing chiefly cellulose acetate and products derived from it, and by a photographic factory at Paris. Before the war there were also film and camera factories in Germany.

The Kodak factories in 1912 and, indeed, ever since were operated by professionally trained chemists and engineers and were equipped with adequate labora-

tories for the control of manufacturing processes, the analysis of raw materials, the testing of finished products and the development of improved processes and products. The new laboratory was not intended to supersede the works laboratories, nor primarily to give service to the factories. It was to study the scientific foundations of the photographic process, to develop if possible entirely new photographic materials, and to discover new applications of photography.

It was decided to erect a new building for the laboratory, and the site assigned was in the centre of the Kodak Park Works, one of the original buildings of the plant being demolished and its site used for the new laboratory. The building was a steel structure having three stories and a basement with a total floor area of approximately 25,000 sq.ft. The basement was devoted to the necessary engineering equipment and to rooms for the preparation of photographic emulsions. The emulsions were made by standard methods and used for the manufacture of colour-sensitive dry plates—the plates that had been made in England by the firm of Wratten and Wainwright, Ltd. These panchromatic plates were thus made for the first time in the United States. This association of manufacturing with the new laboratory seems now a little curious, but at the time it had the definite advantage that the laboratory was able to make its own materials to a considerable extent, and many advances in the preparation of new types of photographic materials came from close association with the manufacturing department.

The laboratory was completed at the beginning of 1913, and the original staff consisted of twenty people, among whom were P. G. Nutting, a physicist who had established himself as an authority in physical optics at the Bureau of Standards, and his assistant, L. A. Jones, who on Nutting's departure in 1917 became chief physicist of the laboratory, a position that he still occupies. The head of the department of physical chemistry was S E. Sheppard, who has continued in that position ever since. Others who have been with the laboratory from the beginning are J. G. Capstaff, who has been responsible for many of the important photographic developments in the laboratory's history, and J. I. Crabtree, who has been in charge of the work on photographic chemistry.

The growth of the laboratory has been continuous, and in spite of the use of the adjoining buildings it was necessary in 1930 to build an entirely new laboratory, to which the existing work was transferred in 1931. The old laboratory building was converted into a laboratory for research work on photographic emulsions, and since the transfer of plate and filter manufacturing to factory departments, the whole of this department has been occupied with research related to the production of photographic materials.

In 1937 a new wing was added to the main laboratory. This building is therefore now an L-shaped building having a front of 180 ft. and a wing 200 ft. from front to back, both sections being 60 ft. deep. Six stories and a basement fully occupied with workrooms provide a total floor space of 152,000 sq.ft. The space is inadequate for our present requirements, and it is planned to build a second wing giving an increased floor area of approximately 60%.

The growth of the laboratory is shown in the following table:

year	total floor area in sq. ft.	size of staff	annual cost ($)
1915	24,500	40	126,745
1920	29,500	88	338,680
1925	39,600	92	397,449
1930	48,700	159	618,503
1935	140,100	210	952,397
1940	205,600	392	1,923,223
1945	207,100	413	2,457,463

From the beginning, the research laboratory has been independent of the departmental organization of the company, and the director has been responsible only to the president and general manager. This was Mr Eastman's proposal originally, and there has been no reason to change it. I became responsible for the conduct of the whole scientific work of the laboratory, including the preparation of the budget and the distribution of the sum allotted to different fields of work, and I was made a director of the company in 1923 and a vice-president in 1934. No approval is asked for specific projects, nor is any report made as to the distribution of the work. The justification for the expenditure has always depended upon the results obtained, though it was understood at the beginning that the laboratory would not be expected to show any justification of its value for a number of years and that it would probably be about 10 years before the results from the laboratory were of financial importance to the company. This assumption proved to be correct, and after the laboratory had been in operation for about 10 years, the materials that it had developed were of considerable importance among the sales of the company and have continued to increase in importance until, at the present time, almost everything that the company makes has in some way been affected by the work of the research laboratory.

The work of the laboratory can be divided into three main sections. About 25% has been on the fundamental science of photography, the theory of the photographic process, about 50% has been on practical photography, and about 25% in the fields of pure chemistry and physics not directly connected with the theory of photography. Thus the study of the theory of photography starts with the physical chemistry of gelatin—its colloid structure, the measurement of its physical properties, its swelling and shrinking, its adsorption to silver halides, and the absorption that it has for other substances. Next, it involves the study of the silver halides—their crystalline form and particularly the distribution of the sizes of the different crystals formed in precipitating an emulsion. The whole subject of the frequency distribution of silver halide grains was studied intensively in the years following 1920, and the subject excited interest both in England and in Sweden at that time. A practical method of determining the frequency distribution of silver halide grains was finally worked out.

In 1925 S. E. Sheppard discovered that the sensitizing action of gelatin for silver bromide, which had always been a mystery, depended upon the presence in the gelatin of sulphur compounds, among which were derivatives of mustard oil, presumably derived from the food of the animals. Following this discovery, he and his collaborators showed that the formation of the latent image depended upon the existence of concentration centres, in which the products derived from the action of light on the silver halide crystal could be concentrated. The accepted theory of the formation of the latent image was developed by Gurney & Mott at Bristol, and their work on the latent image was supplemented by a great deal done in the Kodak Research Laboratories on the effect in forming the latent image of the intensity of light and of the temperature at which the exposure takes place.

A problem at least as difficult as that of the nature of the latent image has been the method by which the silver bromide crystal is converted into silver when it is treated with a developer after exposure to light. There existed a number of theories on the physical chemistry of development, conflicting in nature and none of them with very satisfactory evidence. In 1940-1, however, it became possible to apply the electron microscope to the developed silver image of a photographic material. The grains of silver are too small for their structure to be seen under the ordinary microscope, and it had been generally assumed that they had a coke-like structure. The electron microscope showed that their structure is filamentary and thus at once changed the whole picture of the nature of development, since it is now clear that the filamentary silver is formed *in situ* from the solid silver bromide crystal without any solution of the silver halide There are still doubtful points in relation to the nature of the attack of the developer upon the silver halide crystal, but the matter is rapidly being clarified. The process of development in the formation of a photographic image is, however, very complicated. A complex solution having usually some solvent properties is acting on a fairly thick layer of gelatin containing a number of layers, at different depths, of silver halide crystals that have been given varying exposures.

The measurement of the relation between the developed silver and the exposure given to light and the optical density obtained after development is the branch of photographic study known as *sensitometry*. Work in this field dates from the paper by Hurter & Driffield published in 1890, in which they introduced the use of the *characteristic curve* of a photographic material. In this curve (figure 1) the optical density, $D = \log_{10} 1/T$, is plotted against the logarithm of the exposure. Through a considerable range of exposures (BC in figure 1) the density is proportional to the logarithm of the exposure. Hurter & Driffield termed the exposure corresponding to the intercept of this straight-line portion of the curve on the exposure axis the *inertia* and used its reciprocal as a measure of the sensitivity of the material. They found that under certain controlled conditions this value was independent of the time of development. Thus, for the straight-line portion,

$$D = \gamma \, (\log E - \log i),$$

where γ is the slope. γ, which increases with development to a limit dependent on the material, is termed the *development factor*. In the early days of photographic research the determination of the characteristic curve was a slow and laborious operation. Improvements in the apparatus used for exposure and development and especially the use of automatic photoelectric densitometers have greatly lightened the burden. In the Kodak laboratories thousands of sensitometric curves are now plotted each day.
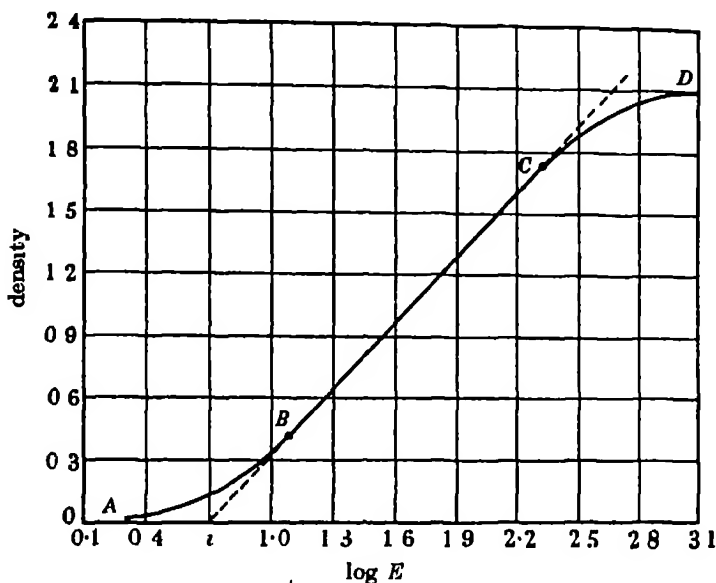
FIGURE 1. Characteristic curve of a photographic material.

From the study of the relation between the exposure and development has come an investigation of the reproduction of the tones of the original subject effected in the picture. The solution generally used is shown in the graphic diagram (figure 2) designed by L. A. Jones about 25 years ago. The brightnesses of the original subject are laid out on the abscissa, and the sensitometric curve of the negative material is plotted in the lower right-hand quadrant so that the intercepts of the perpendiculars on this curve give the translation of the tones of the original into the negative. A similar projection on to the sensitometric curve of the positive material—film or printing paper—which has been plotted in the bottom left-hand quadrant gives the densities of the positive material, and these in turn can be translated into the transparencies of the positive material. Then if these transparencies are projected, using a line in the upper left-hand corner to allow them to form intercepts with the projections of the original brightnesses, we obtain a curve which, if the tone reproduction were perfect, should be a straight line. The distortion of the tone reproduction that has occurred in the process is shown by the departure of the curve from a straight line.

The developed photographic image must be considered not only in its reproduction of the tones of the original subject but in relation to its resolving power, sharpness and graininess, all of which are dependent upon the structure of the

FIGURE 2. Diagram showing the reproduction of tones by the photographic process. *A*, characteristic curve of the negative material; *B*, characteristic curve of the positive material; *D*, curve showing the tone reproduction obtained; *E*, curve showing ideal tone reproduction. The straight line *C* is an aid to projecting points from *B* on to *D*.

developed image and therefore, in turn, upon the structure of the photographic material. The measurement of resolving power, of sharpness, of granularity, and of the subjective graininess of the image has been studied for many years, and

though our knowledge of these subjects is increasing, there is still much work to be done.

In 1926 the motion-picture industry decided to record sound upon film and to print the sound record on the edge of the positive print, which by scanning with a photocell would give a reproduction of the sound. The accuracy of the reproduction and the distortion introduced by the photographic process at different frequencies involve a new application of photographic sensitometry and the theory of tone reproduction.

One of the most important fields of photographic work in the last 40 years has related to the sensitizing of photographic materials by means of dyes. The practical work in this field will be discussed shortly, but mention should be made of the study of the nature of the colour-sensitizing process. Only a limited number of dyes sensitize effectively, and the reason that one dye sensitizes and another does not is only now beginning to be understood. The matter is connected with the structure of the dye and with the nature of its adsorption to silver halide. A great deal of work has been done on this subject, especially in recent years.

Let us turn from this work on the science of photography, which corresponds to about a quarter of the work of the laboratories, to the broad field of practical photography, which occupies nearly half the total energy of the laboratory staff. The biggest single division of the laboratory is that devoted to the making of photographic emulsions, both those used for film and those to be coated upon paper. Photographic emulsions are made by dissolving halides—primarily bromide or chloride—in a weak solution of gelatin and then running a solution of silver nitrate into the mixture until most of the halide is converted into the silver salt. In the case of paper emulsions, which are generally made of silver chloride, the operation is usually complete after the silver nitrate has been added, and the quality of the printing paper depends upon the choice of the halides and of addition compounds and on the rate and temperature at which the precipitate is formed. In the case of film emulsions it is necessary to remove the greater part of the soluble salts from the emulsion in order to prevent crystallization, which would spoil the surface of the coating. To do this sufficient gelatin is added to enable the emulsion to set as a jelly, and it is then cut into fine shreds and washed in cold water to extract the soluble salts.

The whole technique of emulsion making is a complicated art, in which practice is in advance of knowledge of the chemical and physical conditions which control the results. Whereas a great deal has been done to reduce this art to a science, nevertheless in a practical industrial laboratory the development of the art itself cannot be neglected, and a large part of the work in the Kodak Research laboratories has been applied to the advancement of the art. As a result, the photographic emulsions both for film and paper have been steadily improved throughout the years. The high-speed emulsions particularly have been increased in sensitivity and decreased in their graininess and in their tendency to fog—that is, to develop black silver in the unexposed portions. The keeping property also of

the films has been greatly improved. The emulsion laboratories work very closely with the manufacturing departments, and members of the staff of the laboratories are often transferred to the manufacturing departments.

Associated with the emulsion-making laboratories are those dealing with the manufacture of gelatin. Like emulsion making, gelatin making is an art rather than a science. The Eastman Kodak Company manufactures the gelatin that it uses, and the study of possible improvements in the manufacture of gelatin is carried out in association with the emulsion research laboratories.

The emulsion laboratories also deal with the preparation and application of optical sensitizing dyes. In 1916 it became necessary to manufacture dyes that had previously been purchased from Germany, these dyes belonging to the cyanine series and being derived from quinoline. This was accomplished in England by W. H. Mills at Cambridge and in Rochester by H. T. Clarke then in the organic division of the research laboratory.

Work on new dyes was carried on after the war, but not much progress was made until the structure of these dyes had been elucidated by the work of Mills and his collaborators. Pinacyanol, for instance, which had been discovered by Homolka in 1904, was found to have the structure shown in figure 3, the dye being termed by Mills a *carbocyanine* and having three methine groups in the chain joining the two nuclei. Cyanine dyes are known with chains containing 1, 3, 5, 7, 9 and 11 methine groups, and they can be formed from many heterocyclic nuclei. After 1929 very rapid progress was made in the synthesis of new dyes by F M. Hamer in our Harrow laboratory, L. G. S. Brooker at Rochester, J. D. Kendall in the Ilford laboratory, and W. Konig and others in Germany. These new sensitizers were superior to the existing dyes both in their sensitizing power and in their freedom from detrimental effects upon the emulsion. By means of them it was possible to prepare very much improved panchromatic materials, which made an almost revolutionary change in the art of photography, so that the period from 1925 to 1935 was one of rapid change in the development of photographic materials. All this work arose primarily from the discovery of the new sensitizing dyes.
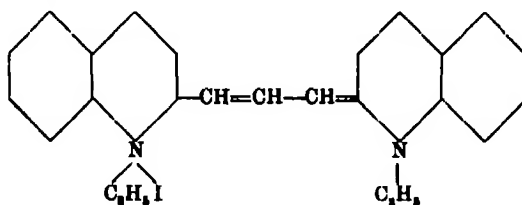

FIGURE 3. Structure of pinacyanol iodide.

The work of the emulsion research laboratory has been of great value in relation to the cinema. In motion-picture photography, the high-speed panchromatic negative film, on which all the motion pictures are taken, arose directly from the work of the laboratory on emulsions and on sensitizers. About 1925 the experts in

motion-picture photography realized that by the use of panchromatic film it should be possible to use tungsten lamps in the studios and thus save a good deal of difficulty caused by the lamps then employed. This was definitely of importance when sound recording was introduced, though at the present time arc lamps are used in studio work, having been improved and silenced to make their use possible.

The positive pictures projected in the theatre used to be printed directly from the original negatives. About 15 years ago, a method of making duplicates of these negatives was introduced, and at the present time a great deal of the printing is from duplicate negatives, the original negative being retained in good condition for future use.

One of the most important introductions into photographic practice has been amateur cinematography. Work on this was started in 1920 It was decided that not only should a small camera and projector be made but that a reversal process should be employed so that the film used in the camera was itself transformed into the positive for projection. The development of the first film to a negative and the printing of a positive were thus avoided. J. G. Capstaff invented an improved reversal process, to which later was added automatic control of the second exposure by photocells, as a result of which machines were designed for continuous processing of the film and transforming it into a positive ready for projection.

By 1932 the development of the new sensitizers made possible the design of an especially fine-grained panchromatic film, on which pictures could be taken only one-fourth the size of the 16 mm. pictures. These little images, having only one-twenty-fifth the area of a standard motion picture, give adequate resolving power for home use. In order to diminish the cost of the film and of its processing, two pictures are exposed side by side on 16 mm. width film and then slit and the 8 mm. wide films are joined end to end for projection.

The 16 mm. film originally designed for amateur cinematography has been applied to documentary photography and used for the photographing of records. During the war, this film was applied to the air mail, and approximately , 1,300,000,000 letters were transmitted to and from the British and American forces as images on rolls of film.

After the synthesis of the new and improved sensitizers, it became possible to realize a new system of photography in colour. In colour photography it is necessary to take three photographs—one by each of the three primary colours—red, green and blue-violet—and to print these photographs in register, using dyes of colours complementary to those by which the picture was taken, so that the picture taken by red light is printed in a cyan, that is, a blue-green dye, the one taken by green light is printed in magenta, and the one taken by blue light is printed in yellow. It had been suggested that this result could be obtained by coating three emulsion layers superimposed on a film base and then converting each image into a dye image by including in the layer a coupler which would form a dye with the developer oxidation product formed by the reaction of the developer with the silver bromide.

The realization of such proposals depended upon the discovery primarily of sensitizing dyes which would not wander from the silver bromide to which they were attached, so that the coating of a second emulsion on the red-sensitive layer would not enable the red sensitizer to leave the silver bromide of that layer and wander into other layers. The sensitizing dyes known before 1930 wandered badly, and only when large numbers of dyes became available could those be selected which would stay satisfactorily in their own layers. Work was started in our laboratories on the realization of this system of colour photography, with the result that in 1935 the first multilayer colour film was placed on the market under the name *Kodachrome*. From this has followed a very large and very rapid development of materials which make it possible for any photographer to take colour photographs with little more effort than that involved in the taking of black and white pictures.

Special photographic plates for use with X-rays had also been made by Wratten and Wainwright, and one of the first things that the laboratory at Rochester did was to introduce similar plates on the American market. The introduction of double-coated X-ray film in 1917 transferred the interest of radiographers from plates to films, and during the first World War a very great use was made of radiography by the military medical authorities. Modern X-ray technique, in fact, dates from that period, and in the laboratory work has been done steadily throughout the whole of its history on the improvement of X-ray materials, both the photographic materials and the intensifying screens. During the second World War, the use of X-rays for medical purposes was, of course, very great, but, in addition, X-rays of high penetrating power were used for the inspection of manufactured products such as parts of airplanes, shells and a multitude of other munitions.

From the beginning, the laboratory has carried out work on the design and computation of lenses. About 10 years ago, work was started on the basic theory of lens optics, and a number of scientific papers have been published in this field. In connexion with this work, it became obvious that a much greater advance could be obtained if new glasses could be found. A suggestion made to us by G. W. Morey was followed up and glass is made with heavy and comparatively rare elements, such as lanthanum, tungsten and thorium, with which a much higher refractive index for the same dispersion could be obtained than was available in the ordinary crown glasses. One of the most useful of these glasses has a refractive index of 1·745 and a ν value of 46. They were made by a new technique, the melts being made in platinum crucibles in the laboratory by 1941, but the demand for the production of lenses for aerial photography so stimulated production that by 1943 the laboratory was making over 1000 lb. of the rare element glass a month. The work was then transferred to a new factory, which raised the production to several tons a month.

Work on optical glasses has been continued and a great number of new glasses have been discovered, but the commercial production of them will take several

years. The work has shown very clearly, however, that the range of optical glasses is by no means limited to those which had previously been conceived.

In addition to the work on photographic theory and on practical photography, the Kodak laboratories have continually strayed into fields of research in applied chemistry. Their necessary interest in chemistry revealed fascinating bypaths, and owing to the lack of strict control and planning in the laboratory some of these bypaths have been pursued far beyond the point which any planned programme would have considered justifiable and have led to some rather remarkable developments.

In the department of organic chemistry, for instance, experiments were started a number of years ago on the treatment of cellulose with nitrogen peroxide. The original idea was that treatment with nitrogen peroxide might serve to facilitate the nitration of cellulose, a matter of considerable importance to our company, which makes its own nitrocellulose for film base. Actually, however, it was found that nitrogen peroxide does not nitrate cellulose to any appreciable extent. Instead, it oxidizes the material and produces a carboxyl group in the structure. This, of course, makes the cellulose soluble in weak alkali. Oxidized cellulose made in this way has some interest for our photographic applications, but by far its most important use is for surgical dressings, since the oxidized cellulose is soluble in blood plasma and can therefore be left in a wound or used for dressings for burns, from which it can be detached without difficulty after the wound begins to heal. The production of oxidized cellulose for those applications has now been undertaken on a commercial scale.

In 1918 we were greatly troubled by our inability to get synthetic organic chemicals for laboratory use, most of the chemicals used in America having been previously obtained from Germany. After discussing the matter with a number of chemical firms, we decided that it would be a useful service for the research laboratory to manufacture synthetic organic chemicals in small quantities and to sell them. This operation has been very successful. The original laboratories have grown into a large department, and the whole undertaking is now on a profitable commercial basis. Incidentally, the possession of an active department of synthetic chemistry capable of manufacturing quantities of chemicals has been of the greatest value in recent years. The department makes all the unusual chemicals required both for photographic manufacturing and, particularly, for colour photography. The special developers and dye couplers as well as dyes themselves are synthesized, and many tons of these complex chemicals are made each year. The original conception was right. It was a desirable service to make available the synthetic chemicals that the research men needed, but the unexpected profit from the undertaking in the availability of chemicals for our own use has proved most gratifying.

The production of synthetic organic chemicals is by no means the only way in which the Kodak Research Laboratories have been able to repay some of the debt that industry owes to the scientific investigators. As soon as the emulsion research

laboratory was organized, it undertook to prepare a series of special plates for use in spectroscopy and astronomy, and these *Eastman Spectroscopic Plates*, as they are called, have been of the greatest value in astronomical photography. More recently, a series of special plates has been made for recording the tracks of nuclear particles. Some years ago the concentration of the rare stable isotopes of nitrogen and carbon, N^{15} and C^{13}, was undertaken, and chemicals containing high concentrations of these isotopes are now being supplied for use as tracers.

One application of the chemical work of the laboratories has resulted in the development of a very considerable industry. This was in the production of cellulose acetate, on which the laboratories started active work in 1926 and in co-operation with the factory made great improvements in the process. In 1932 the company decided to transfer its manufacture of cellulose acetate to the Tennessee Eastman Corporation, an associated company which had been organized in 1920 at Kingsport, Tennessee, its original work being the distillation of wood for the preparation of organic solvents. The Tennessee Eastman Corporation since 1932 has developed a major industry in cellulose acetate yarn and plastics, in acids and solvents, in the manufacture of hydroquinone and chemicals derived from it and of acetate dyes. In order to assist in this important development, the Tennessee Eastman Corporation since 1933 has maintained a special section in the laboratory at Rochester from which have come many of the developments now in production in Tennessee. The head of that department has now transferred to Kingsport as director of research for the Tennessee Eastman Corporation.

One most unexpected development from the research laboratories arose from an attempt to dry film after packing by the use of a high vacuum. K. C. D. Hickman, who undertook this work, became interested in the efficiency of the vacuum pumps and of the gauges that he was using, and he finally drifted off into the study of distillation in high vacua, in which work he was unknowingly duplicating that done by Burch at the Metro-Vickers Laboratory. From this has come an entirely new industry, in which vegetable and animal oils and, particularly, fish oils containing vitamin A and vegetable oils containing vitamin E, are stripped of their vitamins by very large centrifugal stills in a high vacuum. This work, which started a little more than 10 years ago in the laboratories, is now operated by a flourishing company in Rochester, known as Distillation Products, Inc. The company also manufactures a considerable range of apparatus for work in high vacuum.

Our latest deviation from the fields of photography—one undertaken entirely in the hope that 'something may turn up' and without any definite plan of work, like the work on synthetic chemicals or on high vacua, is a study of the properties of phosphors. Phosphors behave in a manner parallel in many respects to the silver halides. They are of interest in connexion with the properties of solids, and we have recently started a long-term study of these materials.

The research work of the Eastman Kodak Company has never been confined to the central laboratories at Rochester. The expenditure, in fact, on the central

laboratories is only about a quarter of the total expenditure on research, experiment and development throughout the companies. Before the research laboratory was established, the works laboratories were engaged in a good deal of experimental work, and this has continued. Each department of the works has facilities not only for the control of its product, but for the development of new products and even for a certain amount of scientific research

· The same policy of decentralization applies to the research done by the Kodak companies throughout the world. The Harrow Works, for instance, maintains a large research laboratory, in which the work parallels and competes with that of the Rochester laboratory. The same statement for a somewhat smaller scale applies to the French laboratory at Vincennes. Scientific papers from both these laboratories are published in the same way as those from Rochester and are incorporated in our total of published communications. A laboratory at Melbourne, Australia, is also active Among the various minor laboratories, a tropical research station in Panama is worthy of mention.

Since the purpose of the laboratory from the beginning was the production of scientific knowledge, the policies of the laboratory have always been directed toward that end. No attempt is made to anticipate scientific discoveries that may be made or to regulate or organize the direction which the work may take When the scientist selects a field of work, he is left free to exploit it as he sees fit, in consultation primarily with the immediate head of his department, much as a graduate student works in collaboration with a professor, but, since our scientists are skilled in research, with much less direction than is usual in the case of research students. There are many conferences, but they are scientific conferences for the exchange of ideas and are not primarily for the direction of the work. Finally, the results of the work are published as communications in the scientific journals to which they are appropriate We do not publish a special journal. In the beginning it was decided that the issue of special journals by industrial laboratories would inflict an intolerable burden upon bibliographers. Chemical papers are published in the journals of the chemical societies, optical papers in the physical or optical journals. For the photographic papers, we have, especially in the United States, been dependent upon the services of certain journals that have been hospitable to us, notably the *Journal of the Franklin Institute*. To prevent this distribution of papers from producing a dispersion which could not be controlled, we publish annually a volume containing abridgements, of about half their length, of all our scientific publications, and these abridgements, which now include well over a thousand communications, form a convenient reference system.

In addition to the scientific communications, the laboratories publish abstract journals dealing with the whole current photographic literature and have been responsible for a number of books.

The policies pursued in the conduct of our research, vague as they must seem to those who believe in systematic planning and in organization, have been enormously profitable. Over the years the cost of the central laboratory has been less

than 4 % of the net profits of the company after taxes. Many major developments have been evolved with but a trifling cost for research, though naturally the later cost for development and commercial exploitation has been very considerable. The result of a lifetime spent on industrial research has convinced me that the research cost is of no great importance. The commercial success of research and development depends upon the choice of the researches to be developed commercially, and this choice is enormously more important than any limitation of the research work itself. Development to the commercial stage costs at least ten times as much as the scientific work in the laboratory, and it is particularly important that directors of research laboratories should not be so biased by their own interest in the results that they select the subjects for commercial exploitation without sufficient attention to the commercial factors involved. Nor can they escape this responsibility by leaving it to the commercial direction of the company, since in the case of new products it is almost impossible for anybody except the research men to foresee the available markets. The research department must, therefore, be fully and intimately allied with the commercial side of the business, and if the director is to be successful he must be fully acquainted with the whole enterprise section of the management. This is why small companies can do research so profitably. In small companies the research director can be an active part of the management to a far greater extent than is possible in a big organization. As examples of the actual cost of some of our work, the research that produced the system of amateur cinematography on 16 mm. film now used throughout the world on a vast scale cost about £50,000 over a term of approximately 12 years. Our research on colour photography, though it has been far more expensive than that on amateur cinematography, has cost less than 5 % of the sale of colour materials up to date. The total cost of the research that led to the organization of Distillation Products, Inc., was much less than the present annual budget for research and development of that company.

In addition to the new materials and new methods that the laboratory has produced, it has been our constant practice to train men for the operating staff of the company. Members of the laboratory staff leave it each year to go to other departments of the company. Many of the technical leaders of the company to-day in factories, in sales, and in general management have had some training in the research laboratory and often were originally employed on the staff of the laboratory and transferred when it was realized that their interests were practical and administrative rather than scientific. This is not an easy thing to do. The men who are transferred are not those who are ineffective in research. No one whose heart is in science is asked to become an administrator, but the men who are transferred are in many cases the most capable and energetic and valuable men on the laboratory staff. To put these men elsewhere involves a considerable amount of resolution and even sacrifice, but the operation has been incomparably successful. The 'graduates' of the laboratory are not only its best friends in the company; they are also the most capable managers that the company can obtain.

Mees

Proc. Roy. Soc. B, volume 135, plate 13
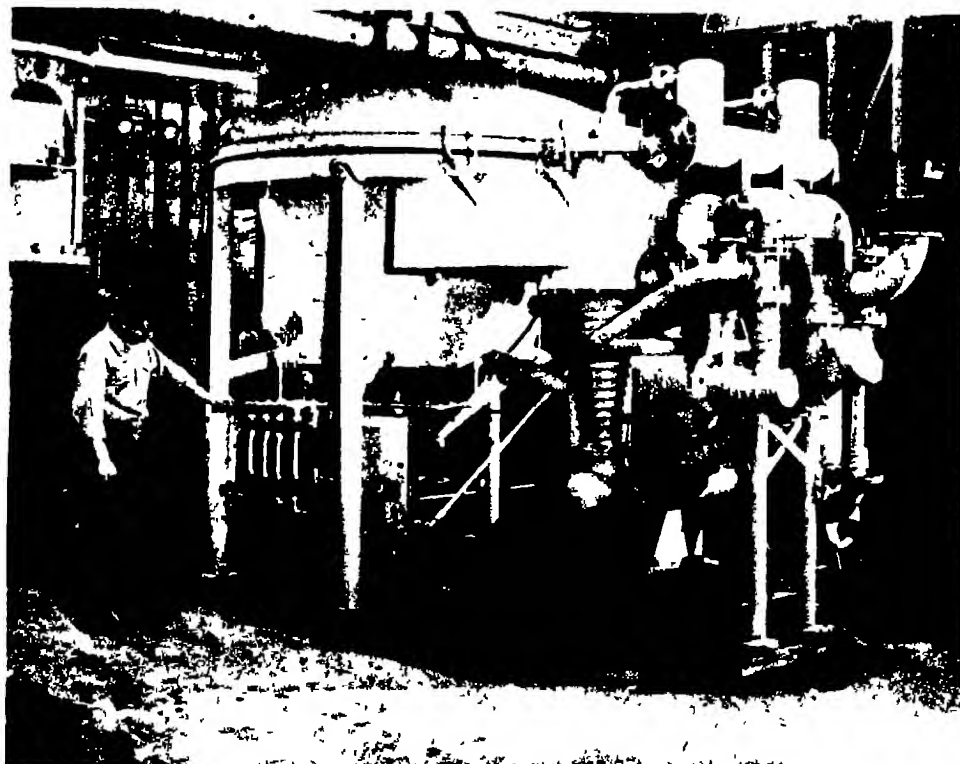
Mr Eastman's vision was correct. The research laboratory, which he founded, has had much influence on the prosperity of his company and, what is more, it has been able to advance the science and art of photography to the benefit of mankind.

REFERENCE

Hurter, F. & Driffield, V. C. 1890 *J. Soc. Chem. Ind.* **9**, 455.

DESCRIPTION OF PLATES 12 AND 13

PLATE 12

Top. Main research laboratory building, Kodak Park, Rochester, N.Y., U.S.A.

Bottom. Five-foot diameter centrifugal 'molecular' still. Distillation Products, Inc., Rochester, N.Y., U.S.A.

The still operates in a vacuum of a few microns. The condensation pumps can be seen at the side.

PLATE 13

Left. Automatic recording densitometer. This plots the points of the characteristic curve.

Right. Experimental machine for coating photographic paper.

CROONIAN LECTURE

The formal genetics of man

BY J. B. S. HALDANE, F.R.S.

Man has obvious disadvantages as an object of genetical study. The advantages are that very large populations are available, and that many serological differences and congenital abnormalities have been intensively investigated.

Some characters are found to obey Mendel's laws with great exactitude. In others the deviations are such as to suggest the existence of a considerable selective mortality, perhaps prenatal. In yet other cases the observations are biased because we only know that we are investigating the progeny of two heterozygotes when the family includes at least one recessive. Statistical methods which eliminate this bias are described.

Still more complex methods are needed for the detection and estimation of linkage. Several such cases have been detected with greater or less certainty, and the frequency of recombination between the loci of the genes for colour-blindness and haemophilia is now estimated at $10 \pm 4 \%$. If the theory of partial sex-linkage be accepted, it is possible to make a provisional map of a segment of the human sex chromosome.

When a gene is sublethal, as are those for haemophilia and achondroplasic dwarfism, its elimination by natural selection is in approximate equilibrium with its appearance by mutation, and the frequency of the latter process can be estimated. The mutation rates at five human gene loci lie between 4×10^{-5} and 4×10^{-5} per locus per generation. These are the only estimates available for vertebrates. The rates per generation are rather higher than those in *Drosophila*, but those per day are so small that much, or even all, human mutation may be due to natural radiations and particles of high energy.

In 1906 Bateson delivered a lecture to the Neurological Society on 'Mendelian heredity and its application to man' in which he described the genetics of brachydactyly and congenital cataract, which are dominant to the normal (the word is used loosely, since the abnormal homozygote is unknown). He suggested that albinism and alcaptonuria were recessive, and he described the laws of inheritance of haemophilia and colour-blindness, though he did not, of course, give the explanation of these laws which is now accepted

In the ensuing 40 years a very large number of pedigrees have been collected, unfortunately with very variable standards of accuracy. These show that more than a hundred different human abnormalities are certainly due to single gene substitutions, and that several hundred more are probably so. For example, Cockayne (1933) listed eighty abnormalities of the skin, hair, nails, and teeth which are probably due to dominant autosomal genes, eighteen to autosomal recessives, and thirteen to sex-linked genes. In over half the cases the evidence is adequate.

On the other hand the genetical analysis of the normal polymorphism of a race such as our own for colour, size, and shape has not gone far. The genetics of eye colour, for example, are far more complex than was originally thought, and stature is undoubtedly determined by a large number of genes, as well as by environmental influences Still less progress has been made in the analysis of the genetics of those differences in skin colour and hair shape which exist between the major human races. However, immunology has revealed a polymorphism existing in all races which was wholly unexpected when Bateson wrote. Its genetical basis is exceedingly simple; perhaps because antigens are direct products of gene action, while pigments are the end products of complex chains of metabolic processes in which many, if not all, of the steps are controlled by different genes, and the processes of morphogenesis are even more complicated. Meanwhile, genetics have developed along many lines, of which three are especially important. It has been shown that genes are material structures located at definite points in the chromosomes. If we can homologize the genes of organisms which conjugate and the 'transforming principles' of bacteria, which can apparently transfer them to one another without conjugation, just as they carry out a communal metabolism, the work of Avery, MacLeod & MacCarty (1944) suggests that genes, at least in some phases of their life cycles, may consist wholly of desoxy-ribose nucleic acid.

We have learned a good deal about the causal chain between a gene and its manifestation. Goldschmidt was a pioneer in this work. You, Mr President, played an important part in the analysis of the genetical control of anthocyanin production in flowers. Beadle, Tatum and others were able to specify the stages in the production of arginine and other essential metabolites controlled by different genes in *Neurospora*. In this country, Grüneberg, in the mouse, and Waddington, in *Drosophila*, investigated the genic control of morphological development.

Finally, a number of workers, notably Dobzhansky, Dubinin, Fisher, Haldane, Teissier & l'Heritier, Tsetverikov, and Wright have investigated the genetics of

populations both practically and theoretically, and they and others have discussed the bearing of their results on the problem of evolution.

All these methods are applicable to our own species. There is, however, a widespread belief that what I may call formal genetics, that is to say the study of heredity and variation, based on the description and counting of individuals, has ceased to be important, and that in future genetics will consist mainly of the study of biochemical and morphogenetic processes controlled by genes, and of evolutionary changes in populations; while the mere enumeration of the results obtained from various matings, and deductions drawn from such enumeration, are no longer of great interest. As I propose to devote this lecture to the pure or formal genetics of man, I may perhaps be pardoned if I state what seem to me to be the legitimate aims of human genetics, and so to justify what some will regard as a reactionary standpoint

The final aim, perhaps asymptotic, should be the enumeration and location of all the genes found in normal human beings, the function of each being deduced from the variations occurring when the said gene is altered by mutation, or when several allelomorphs of it exist in normal men and women. In addition, information would be gathered on the effect of changes involving sections of chromosomes, such as inversions, translocations, deficiencies, and duplications

The number of genes in a human nucleus almost certainly runs into thousands, possibly tens of thousands Each has, so far as one can judge, a highly specific biochemical function The end result of such a genetical study as I have adumbrated would be an anatomy and physiology of the human nucleus, which would be incomparably more detailed than the anatomy and physiology of the whole body as known at present. This end will perhaps be achieved in part by non-genetical methods, such as ultramicroscopic operations on the nuclei of human cells in tissue culture

No doubt one result of such a study will be the possibility of a scientific eugenics, which may bear the same relation to the practices now or recently in vogue in certain countries as chemotherapy bears to the bleedings and purgations of early medicine But other results may be more important A knowledge of the human nucleus may give us the same powers for good or evil over ourselves as the knowledge of the atomic nucleus has given us over parts of the external world.

In this lecture I shall be largely concerned with the localization of genes in human chromosomes. A simple example will show why this is important. One of the common causes of blindness is retinitis pigmentosa. Ten years ago it could be said that in some pedigrees this disease was transmitted as a dominant, in others as a recessive of the ordinary type, occasionally as a sex-linked recessive. In 1936 I argued that some pedigrees showed partial sex-linkage, a phenomenon which I shall describe later. We can now say tentatively that one of the genes, the abnormality of which causes this condition, is carried in that segment of the sex chromosomes of which women possess two, and men only one, another, which may give dominant or recessive mutants, in that segment of the same chromosomes of which both sexes

possess two; while other such genes, how many we do not know, are carried in the other chromosomes. It is reasonably sure that they control different processes. And this is borne out by the fact that the partially sex-linked recessive type is never associated with deafness, while one of the autosomal recessives is so associated

Pathologists will have to work out the aetiology of the different genetical types They can hardly hope to do so until they are distinguished, if, as seems probable, each gene controls a different process. And just as the methods for the cure of bacillary and amoebic dysentery are very different, so it is unlikely that the same therapeutic measures will succeed against diseases, however similar in their symptoms, which are due to different genes They certainly do not do so in *Drosophila melanogaster*. In that species at least four different recessives give eye colours which are scarlet because they lack a yellow pigment found in the normal eye, which is a derivative of tryptophane. The eye colour of the mutant *vermilion* can be made normal by injecting the larvae with kynurenine, for the gene present in normal flies but inactive in *vermilion* flies is concerned in the oxidation of tryptophane to kynurenine. But *cinnabar* flies are not cured, because they cannot catalyze a further stage in the pigment formation, and *cardinal* and *scarlet* flies are not cured because their eye rudiments cannot take up the pigment precursor (Ephrussi 1942). The four genes in question are carried at different loci in three chromosomes.

The first step in formal genetics is to establish that certain characters are inherited in accordance with Mendel's laws, and in particular that segregation occurs in Mendelian ratios.

This is certainly true in many cases where large numbers have been studied Thus according to theory a member of blood group AB produces equal numbers of A and B gametes Table 1* shows that this is the case, the deviation from theory being less than the standard error of sampling In the mating $A \times AB$ the A children are derived from A gametes of the AB parent, the B and AB children from B gametes, and so on Such an agreement implies not only that the two types of gamete are

TABLE 1

	children in group				
parents	O	A	B	AB	total
$O \times AB$	8 !	633	646	3 !	1290
$A \times AB$	0	533	247	312	1092
$B \times AB$	2 !	183	406	232	823
$AB \times AB$	0	28	36	65	129

total A gametes 1609⎫
total B gametes 1647⎭ $(d-1) n^{-\frac{1}{2}} = 0\,648.$
total homozygotes from $AB \times AB$ 64.
total heterozygotes from $AB \times AB$ 65.

* From Wiener (1943), p. 190. This includes all data published since 1931. Before this date only groups of over 250 children are included. This criterion omits three children of AB mother assigned to group O by two workers who had tested seven and nine children of such mothers, and whose findings have perhaps received undue attention.

formed in equal numbers, but that there is no marked selective mortality of either type of zygote. Thirteen children occur in unexpected groups These represent the combined effects of illegitimacy, technical errors, and conceivably mutation or abnormal segregation of chromosomes. Clearly these causes combined will produce results smaller than sampling error.

In the case of the pair of allelomorphic genes which respectively produce the *M* and *N* agglutinogens, there is at least prima facie evidence for very abnormal segregation Where such cases have been investigated in animals, they have so far almost always been found to be due to selective mortality, or in fact natural selection, one genotype having a higher deathrate in the early stages of life than another Taylor & Prior (1939a) found that the progeny from the marriages of heterozygotes which they had examined included a considerable excess of heterozygotes They were convinced that this was not due to technical errors, and found a similar excess in the pooled data of other workers, giving 54·85 % of heterozygous children, the excess being 3·08 times its standard error Wiener (1943) believes the excess to be an artefact due to the use of incompletely absorbed testing fluids, and that no such excess occurs where this error is avoided. Now if the sera used give too many *MN* children they should also give too many *MN* parents Taylor & Prior (1939b) showed that in most series of data the square of the number of *MN* individuals approximates to four times the product of the numbers of *M* and *N*, while a few show a great excess. I have therefore applied their test to all the series in Wiener's table 46, and eliminated cases where χ^2 exceeds four for the parents † I have also omitted two small series containing 40 children between them, and two Japanese and one German series which were not available in English libraries, and have inserted one German series. The results are shown in table 2 The values of χ are calculated by Taylor & Prior's method, a positive value denoting an excess of *MN* over expectation among the parents. It will be seen that there are 54·85 % of heterozygotes The excess is 3·16 times its standard error. The probability of so large an excess or defect by sampling error is 0·0016. If it were due to the systematic use of incompletely absorbed testing fluids, we should expect to find a lower percentage of heterozygotes in those series where χ is negative. The value found is 56·77 %, which is slightly, but not significantly higher The excess of *M* over *N* children is just below twice its standard sampling error, and not statistically significant.

If the numbers of heterozygotes (*MN*) and homozygotes (*M + N*) in the different groups are compared, we find $\chi^2 = 17·21$ for 11 degrees of freedom. Thus $P = 0·10$, and the data are not significantly heterogeneous, as they would probably be if some workers had used faulty methods.

Table 3 shows the totals found for all types of marriage by the twelve authors cited in table 2. Illegitimacy, technical errors, and mutation clearly account for

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† Wiener gives the reasons for excluding Lattes & Garrasi's (1932) data. The other relevant values of χ are· Dahr (1940) +4·51, Hirzfeld & Kostuch (1938) +2·16, Landsteiner & Wiener (1941) +2·002, Wiener & Sonn (1943) −2·20 The last three values may well all be due to sampling error.

very few unexpected classifications. In the case of the $MN \times M$ and $MN \times N$ marriages, the differences between the classes where equality is expected are less than twice their standard errors. It will be seen that the mean number of children examined per $MN \times MN$ marriage was decidedly less than that in the other groups. The figure 2·73 is not of course the mean fertility per marriage, as sterile marriages were excluded, it was not always possible to examine all the living children, and

TABLE 2

authors	number of families	χ	children M	MN	N
Landsteiner & Levine	11	+ 0 017	17	31	7
Wiener & Vaisberg	25	− 0·006	29	58	29
Schiff	33	+ 1 808	18	48	22
Crome	9	+ 0 083	4	10	4
Clausen	70	− 0·777	38	74	28
Blaurock	23	+ 1 002	25	40	25
Moureau	53	+ 0·444	45	102	41
Hyman	32	+ 0 906	10	41	16
Matta*	{ 20 20	− 0·781 − 1·741 }	9	45	10
Dahr & Bussmann	30	− 0 514	38	70	18
Taylor & Prior (a)	56	− 0·349	10	38	8
Holford	34	+ 1 185	24	37	14
total	416	—	267	594	222

* One group in Egypt, one in Glasgow.

TABLE 3. PROGENY OF DIFFERENT MARRIAGES INVOLVING M AND N

parents	number of families	M	MN	N	total	mean children per family
$M \times M$	147	425	3 !	0	428	2 91
$M \times N$	151	1 !	477	2 !	480	3 18
$N \times N$	74	0	0	232	232	3·13
$MN \times M$	397	597	662	4 !	1263	3·18
$MN \times N$	292	2 !	428	483	913	3·13
$MN \times MN$	396	267	594	222	1083	2·73
total	1457	—	—	—	4399	3 02

investigators tend to choose large families. Nevertheless, it suggests that such marriages are less fertile than the average. The shortage of total children and of homozygotes can both be explained if homozygotes have a higher deathrate (probably prenatal) than heterozygotes. The prenatal and infantile fitness of the homozygotes is about 82 % of that of the heterozygotes, so at least 18 % of them must die at an early stage. If there had been 594 homozygotes instead of 489, the mean family size would have been 3·00. The hypothesis of selective death implies that 105, or 2·3 % of a group of 4504, human zygotes were eliminated, probably before birth. If the $MN \times MN$ marriages had been as fertile as the rest, we should have

expected 155 more children from them, making a total of 4659, of whom 3·4 % were eliminated.

This is a substantial fraction of all conceptions, and it would seem that if a scientific study of the problem of human population is to be undertaken, it would be desirable to investigate a group of say 5000 married couples (including sterile couples) serologically, in order to discover whether certain types are less fertile than others, and whether certain human genotypes are eliminated prenatally. It would be essential, in such a study, to tabulate the results of reciprocal unions such as $MN♀ \times M♂$, and $M♀ \times MN♂$, separately. Unfortunately, many of the authors cited did not do so. It is of course possible that Wiener's hypothesis is correct Nevertheless, the matter seems sufficiently important to warrant further study.

In the case of the *Rh* group of genes it is known that certain classes of offspring are killed off because they immunize their mother, and their blood corpuscles are destroyed by her antibodies. Such a mechanism will not explain the results found with M and N. Moreover, the elimination of homozygous offspring of two heterozygous parents would make the equilibrium between the two genes unstable, whereas in fact their frequencies in different peoples are much less variable than those of other genes responsible for serological differences.

Whatever may be the final answer to these questions, I hope I have shown that the exact investigation of the segregation of common genes is not a matter of merely academic interest

I must pass on to the methods which are used in the investigation of the segregation of rare genes When the rare gene is a dominant there are no statistical difficulties provided the gene manifests itself in all heterozygotes, and early in life We cannot possibly expect to find Mendelian ratios for such a character as Huntington's chorea, whose mean age of appearance is about 35 years We should expect to find good results in the case of hereditary skin diseases, which are easily and accurately diagnosed, and mostly manifested at an early age. Table 4 shows the children from unions of affected and normal persons in the sixteen diseases inherited as dominants of which Cockayne (1933) in his classical treatise was able to collect records of over 100 such children. A few of my numbers differ slightly from his totals through the exclusion of doubtful pedigrees. As a result of sampling error we should expect a normal distribution about zero with unit variance of the values of $(d-1)n^{-\frac{1}{2}}$, when d is the number of affected minus that of normal, and n is their sum. There are three aberrant values. The low incidence of neurofibromatosis may possibly be accounted for by its variable age of onset and sublethal character. Some individuals carrying the gene may have died prenatally, others may not yet have developed it when observed. Hypoplasia of the enamel is due to genes in at least two different chromosomes (Haldane 1937) and therefore presents complications. Tylosis, which is an abnormal thickening of the skin of the palms and soles, generally develops in the first year of life. It seems to present a definite exception to the usual rules, and demands further investigation. The similar anomalous cases which occur in the literature of dominant abnormalities of other organs are easier to explain by faulty diagnosis There seems

no reason to doubt that the segregation of most human dominant abnormalities follows Mendel's laws

The ratios in which a gene pair segregates cannot be obtained so simply when one allelomorph is fully recessive. This is due to the fact that the compilation of a pedigree introduced a certain bias. The bias may be of a very simple kind. Birch (1937) in Chicago and Andreassen (1943) in Copenhagen collected 146 pedigrees of haemophilia, a sex-linked recessive condition, transmitted to and from males

TABLE 4. PROGENY OF INDIVIDUALS AFFECTED WITH DOMINANT ABNORMALITIES OF THE SKIN, HAIR, NAILS, AND TEETH

abnormality	affected	normal	$(d-1)n^{-\frac{1}{2}}$
piebaldness	133	118	+0·88
cutaneous xanthomatosis	98	111	−0·83
telangiectasis	320	302	+0·43
epidermolysis bullosa simplex	193	163	+1·54
epidermolysis bullosa dystrophica	147	181	−1·82
monilethrix	92	89	+0·15
porokeratosis	70	91	−1·58
tylosis plantaris et palmaris	594	483	+3·35
ichthyosis vulgaris	86	98	−0·81
alopecia congenita	130	118	+0·70
onychogryphosis	242	253	−0·45
hypoplasia of enamel	84	50	+2·81
neurofibromatosis	115	160	−2·61
naevus aplasticus	53	61	−0·66
fistula auris	63	60	+0·18
angioneurotic oedema	182	206	−1·17
total	2602	2544	+0·79

TABLE 5. SONS OF HAEMOPHILICS' DAUGHTERS

	families	normal sons	haemophilic sons	
mothers of patients	17	11	26	(−17)
other mothers	26	25	23	+1?
total	43	36	49	+1?(−17)
				= 32 + 1?

through females. Each pedigree began with a patient whose relatives were then traced. In order to verify that the condition is due to a single gene we must show, among other things, that the daughters of haemophilics bear equal numbers of normal and haemophilic sons. If we study the daughters of haemophilics in the pedigrees we find a considerable excess of haemophilic sons. However, a further analysis (table 5) shows that this excess is confined to the mothers of the patients from whom the compilation of the pedigree started.

The reason is simple. The mothers of patients were investigated because the patient was discovered to be haemophilic. Hence at least one of their sons must have been haemophilic. The other daughters of haemophilics may have borne no

haemophilic sons, indeed one was fortunate enough to bear three normal sons and no haemophilic. We can allow for this bias by subtracting one haemophilic from each family including a patient. The total then becomes 36 normal sons, with 32 haemophilics and one doubtful, a very good approximation to equality. A similar but more complicated analysis shows that about half the sisters of haemophilics transmit the disease A neglect of this elementary point has led to the most remarkable conclusions as to the fertility of human stocks afflicted with hereditary disease. For if a character is passed on to half the children of an afflicted person, it will not be recognized as hereditary unless at least one child possesses it We shall thus exclude all families of no children, half the families with only one child, a quarter of those with two, and so on, thus giving a wholly false impression of the fertility of such stocks Where, on an average, the character only appears in one-quarter of the children, the exaggeration is still greater.

Unfortunately, the very simple type of correction which was applicable to the pedigrees of haemophilia cannot always be applied

Consider a recessive character such as albinism or amaurotic idiocy which, by analogy with animals, is to be expected in one-quarter of the progeny of unions between two heterozygotes of normal appearance We have in general no evidence that a pair of parents is heterozygous, except that they have produced at least one recessive. We cannot therefore study the progeny of a number of pairs of known heterozygotes, as we can in animal experiments We can only study the progeny of those pairs which have produced at least one recessive.

Clearly the frequency of recessives in such sibships* is greater that the expected quarter. For it is 100 % in sibship of one, and over 50 % in sibships of two. The method for assessing the frequency p which would be found in a very large sibship from data on small ones depends on how the data are collected

Let a_{rs} be the number of sibships of s members, of which r are abnormal.

Let $t_s = \sum_r a_{rs}$, i e. the total number of sibships of s members.

Let $N = \sum_{r,s} a_{rs}$, i e. the total number of sibships.

Let $R = \sum_{r,s} (r a_{rs})$, i e. the total number of abnormals.

Let $S = \sum_{r,s} (s a_{rs})$, i e. the total number of sibs.

Let $q = 1 - p$.

Now consider two ideal cases. In the first case a whole population is surveyed, and all sibships containing at least one abnormal are tabulated This is possible in a small European country. Thus Sjögren (1931) probably tabulated over 90 % of the Swedish families in which a case of juvenile amaurotic idiocy had occurred in the twentieth century. In this case the estimate of p is given by.

$$\frac{R}{p} = \sum_s \left[\frac{s t_s}{1 - q^s} \right],$$

* The word sibship means a set of siblings, that is to say brothers and/or sisters.

and its standard error is given by:

$$\sigma_p^{-2} = \frac{R}{p^2 q} - \frac{1}{q^2} \sum_s \left[\frac{s q^s t_s}{(1-q^s)^2} \right].$$

Unfortunately, this cumbrous equation, due to Haldane (1938), can be shown to yield a result with a smaller standard error than a simpler one due to Weinberg. Perhaps a quicker but equally efficient method may be devised.

In the second ideal case all children leaving school in a certain year, or better all children born in a certain 6 months, are examined. The sibs of all abnormal children are tabulated. Clearly if a sibship contains three abnormals, it is three times as likely to be tabulated as if it contains only one, and so on, apart from an obvious correction for twins. In this case the estimate of p is

$$p = \frac{R-N}{T-N}, \quad \text{and} \quad \sigma_p^2 = \frac{(T-R)(R-N)}{(T-N)^3},$$

an elegant result due to Weinberg (1927).

Applying these methods to Pearson, Nettleship & Usher's (1913) collection of 411 sibships from normal parents including at least one albino, 864 out of 2435, or 35·48 %, were albinos.

Applying the first correction

$$p_1 = 0\cdot3082 \pm 0\cdot0107,$$

applying the second $\qquad p_2 = 0\cdot2238 \pm 0\cdot0092.$

The Mendelian value of $\frac{1}{4}$ lies between these two estimates, and there is reason to think that if an exact correction were possible, the Mendelian ratio would be found. A simple example will show the need for other corrections. According to Andreassen (1943), in the year 1941 there were 1,820,000 males in Denmark, of whom 81 were haemophilic. Almost all their families were investigated, and valuable results were obtained. However, haemophilics have a much shorter average life than ordinary males (about 18 years in Denmark). So a large fraction, probably the majority, of the haemophilics born between 1910 and 1930 were dead by 1942. A family into which three haemophilics were born in that time was more likely to contain a living haemophilic in 1942 than one into which only one was born But it was not exactly three times as likely. If half the haemophilics had died, and there was no correlation in the age of death between haemophilic sibs, it was 1·75 times as likely Hence the true value of p would lie somewhere between the two extreme estimates. Special methods could and should be developed for such cases

They are important because they offer a possibility of investigating selective prenatal death, of verifying the general applicability of genetical principles to man, and of developing, in comparatively simple cases, the quite peculiar statistical methods which are required when the genotypes of parents must be deduced from the phenotype of the children, with an accuracy which increases with the size of the

sibship. These methods may be said to play the same part in human genetics that standard culture methods play in animal and plant genetics. Without them qualitative conclusions may be drawn, but quantitative work is impossible in the case of many genes.

We now pass to the methods for the location of genes on the human chromosomes A serious beginning has been made with the mapping of two sections. One is the segment of the X chromosome which is responsible for sex determination, in which the ordinary sex-linked genes are located The other is the segment common to the X and Y chromosomes. With regard to the remaining twenty-three there is fairly good, but never conclusive, evidence, for the compresence in one chromosome of two genes. For each type of location appropriate statistical methods have been developed
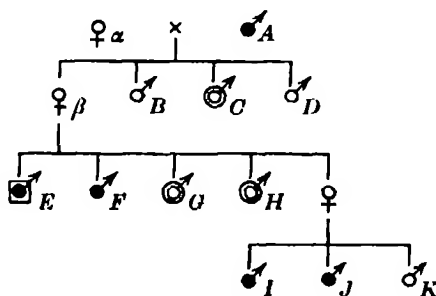
FIGURE 1. ♂, normal male. ⊘♂, deuteranopic male, not haemophilic, ♂, haemophilic male, vision untested, ⊡♂, haemophilic trichromatic male.

Let us begin with the differential segment of the X. A large number of sex-linked recessive genes have been located there. The mode of inheritance of the characters determined by them is highly characteristic. The abnormality determined by any one of them is far commoner in men than women. It is not transmitted from a father to his son. But it occurs in about half the sons of heterozygous women, who include the daughters of affected men The X chromosomes of *Drosophila* species have been mapped by studying the segregation of genes in the progeny of mothers who are heterozygous for two or more pairs of sex-linked allelomorphs.

Cytological studies have shown that the maps so obtained depict real material structures, as X-ray diffraction and reflexion have shown that the structural formulae of the organic chemist depict real objects.

At one locus in the human X chromosome abnormalities are very common About 8 % of all men are colour-blind or anomalous colour-matchers. Hence if we wish to estimate the percentage of recombination between the loci of haemophilia and colour-blindness we must search for colour-blindness among haemophilics and their brothers. Seventeen pedigrees are known in which both abnormalities are found. Of the total information available from them, about a third was collected by Bell & Haldane (1936), another third by the Dutch physician Hoogvliet (1942) and the remainder by five others.

The method employed can be illustrated by two simple examples. Figure 1 shows a pedigree in which A was a haemophilic, while α carried the gene for colour-blindness in one of her two X chromosomes. She also gave it to β, who had two colour-blind sons. So β had the genes for these two defects in different chromosomes (in the *trans* position, if we like a metaphor from organic chemistry, or in repulsion in Bateson & Punnett's terminology). Of β's three surviving sons, one was a haemophilic trichromat; two were colour-blind, but had normal blood Now if x is the frequency of recombination between the two loci concerned, the probability of β producing just these three sons is $(1-x)^3$, given that she had one haemophilic and two non-haemophilics If one of the non-haemophilics had had normal vision it would have been $x(1-x)^2$, and so on. On this pedigree taken alone the best estimate of x is clearly zero.

Figure 2, which is part of Bell & Haldane's (1937) pedigree A, of 98 members, raises a rather more subtle problem.
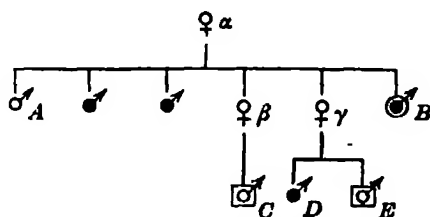
FIGURE 2. σ, normal male; σ, haemophilic male, vision untested; σ, trichromatic male, not haemophilic; σ, haemophilic deuteranopic male.

This was solved approximately by Bell & Haldane (1936), but my colleague Dr Smith has produced a more accurate method (Haldane & Smith 1947). We ask what is the probability that seven persons related in the manner shown should have just these phenotypes. If p_c and p_h are the frequencies of the genes c and h for colour-blindness and haemophilia, we ask what is the probability that α should have been heterozygous for both of them Clearly it is $4p_c(1-p_c)p_h(1-p_h)$ If α was doubly heterozygous and her husband normal, there are eight possible sets of events in the formation of ova by α, β, and γ which could have given the observed results. They are shown in figure 3 and the probability of each is given, putting $y = 1-x$. It is the product of five factors representing the probabilities of the formation of five different eggs. These are shown in each case Since γ had a haemophilic son who died in infancy we know that she received the gene h from her mother. This excludes sixteen other possibilities whose probability is zero. It does not exhaust the possibilities. For though we can be sure that neither α nor her husband was haemophilic, we cannot be sure that one or both of them was not colour-blind. So the total probability has two more terms, each containing p_c^2. It is

$$\tfrac{1}{8}p_c p_h[3(1-p_c)(1-2x+4x^2-4x^3+x^4)+p_c(x^2-x^4)].$$

Since p_c is of the order of 0·01 it can in practice be neglected in this case, but not in all cases.

The corresponding expression for the pedigree of figure 1 is

$$2^{-11}p_c p_h (1-x)^3.$$

These probabilities are of course small, partly because the genes in question are rare, partly because the particular pattern of segregation found is one of a vast number which are possible, like the 635,013,559,600 equiprobable bridge hands. However, each is maximal for some value of x between 0 and 1 inclusive, in the cases considered, for $x = 0$. In other pedigrees a cross-over has occurred, i.e. the genes c and h have entered a woman in one gamete and left her in different ones or vice versa. In these the polynomial is maximal for some other value of x. The product of the seventeen polynomials derived from the different pedigrees is maximal when $x = 0.098$, and we may estimate the frequency of recombination between the two loci as $9.8 \pm 4.2 \%$.

White (1940) found $64.8 \pm 12.7 \%$ of recombination between the loci of colour-blindness and of myopia with nystagmus, so the genetical map of the human X chromosome is likely to be as long as that of *Drosophila melanogaster*, though probably shorter than that of *Gallus domesticus*.
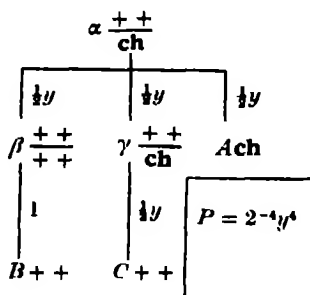
Ten years ago I accounted for the peculiar inheritance of certain characters on the hypothesis that the genes concerned in their determination were located in that segment of the sex chromosomes which is common to the X and Y, and may be exchanged between them (Haldane 1936). Such genes are said to be partially sex-linked.

At that time I put forward the hypothesis with considerable misgiving, but it has been generally accepted, and I shall therefore state it with comparative confidence. Penrose (1946b) has recently given an alternative explanation for some cases which appear to conform to it, but he does not think that this will explain all the cases. First, consider a dominant gene in this segment. If a woman has it, necessarily in an X chromosome, she will transmit it, on an average, to half her children, regardless of sex. If a man has it in his Y, he has inherited it from his father, and will probably transmit it to most of his sons but a few of his daughters. If he has it in his X he has inherited it from his mother, and will transmit it to most of his daughters but a few of his sons. Thus the sex of the affected children of affected males will generally be the same as that of the affected paternal grandparent. The fewer the exceptions, the nearer the locus to the differential segments containing the sex determiners, and (in the X) the loci of such genes as haemophilia.

Only one such partially sex-linked dominant is known, namely retinitis pigmentosa in some pedigrees. Penrose (1946b) has, I think disproved Pipkin & Pipkin's (1945) claim to have found a second such, zygodactyly, or webbing of the toes. It is possible that my own claim in the case of retinitis pigmentosa will equally be disproved.

The location of partially sex-linked recessives is not so simple, but I think some of its results are more certain. Where the parents are first cousins, the sex of the affected offspring is usually the same as that of the paternal grandparent through whom the parents are related. For if this grandparent was a male the father carries

the gene in his Y chromosome, and will transmit it predominantly to his sons, as in figure 4 If she was a female they will mostly be daughters. If the parents are not known to be related, we can only say that in any particular sibship the affected members will be predominantly of one sex, though in all sibships together no such predominance is to be expected, except in the case of very rare conditions where the parents are mostly consanguineous.

$$\alpha \frac{++}{ch}$$

$\frac{1}{2}y \qquad \frac{1}{2}y \qquad \frac{1}{2}y$

$\beta \dfrac{++}{++} \qquad \gamma \dfrac{++}{ch} \qquad A\,ch$

$1 \qquad\qquad \frac{1}{2}y \qquad P = 2^{-4}y^4$

$B++ \qquad C++$

1.

$$x \frac{++}{ch}$$

$\frac{1}{2}y \qquad \frac{1}{2}y \qquad \frac{1}{2}y$

$\beta \dfrac{++}{ch} \qquad \gamma \dfrac{++}{ch} \qquad A\,ch$

$\frac{1}{2}y \qquad\qquad \frac{1}{2}y \qquad P = 2^{-5}y^5$

$B++ \qquad C++$

2

$$\alpha \frac{++}{ch}$$

$\frac{1}{2}x \qquad \frac{1}{2}y \qquad \frac{1}{2}y$

$\beta \dfrac{++}{c+} \qquad \gamma \dfrac{++}{ch} \qquad A\,ch$

$\frac{1}{2} \qquad\qquad \frac{1}{2}y \qquad P = 2^{-5}xy^3$

$B++ \qquad C++$

3.

$$\alpha \frac{++}{ch}$$

$\frac{1}{2}x \qquad \frac{1}{2}y \qquad \frac{1}{2}y$

$\beta \dfrac{++}{+h} \qquad \gamma \dfrac{++}{ch} \qquad A\,ch$

$\frac{1}{2} \qquad\qquad \frac{1}{2}y \qquad P = 2^{-5}xy^3$

$B++ \qquad C++$

4

$$\alpha \frac{++}{ch}$$

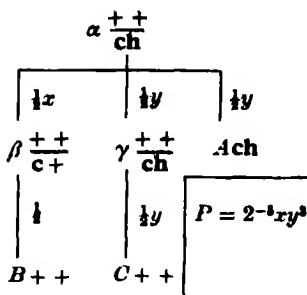$\frac{1}{2}y \qquad \frac{1}{2}x \qquad \frac{1}{2}y$

$\beta \dfrac{++}{++} \qquad \gamma \dfrac{++}{+h} \qquad A\,ch$

$1 \qquad\qquad \frac{1}{2} \qquad P = 2^{-4}xy^2$

$B++ \qquad C++$

5.

$$\alpha \frac{++}{ch}$$

$\frac{1}{2}y \qquad \frac{1}{2}x \qquad \frac{1}{2}y$

$\beta \dfrac{++}{ch} \qquad \gamma \dfrac{++}{+h} \qquad A\,ch$

$\frac{1}{2}y \qquad\qquad \frac{1}{2} \qquad P = 2^{-5}xy^3$

$B++ \qquad C++$

6.

$$\alpha \frac{++}{ch}$$

$\frac{1}{2}x \qquad \frac{1}{2}x \qquad \frac{1}{2}y$

$\beta \dfrac{++}{c+} \qquad \gamma \dfrac{++}{+h} \qquad A\,ch$

$\frac{1}{2} \qquad\qquad \frac{1}{2} \qquad P = 2^{-5}x^2y$

$B++ \qquad C++$

7.

$$\alpha \frac{++}{ch}$$

$\frac{1}{2}x \qquad \frac{1}{2}x \qquad \frac{1}{2}y$

$\beta \dfrac{++}{+h} \qquad \gamma \dfrac{++}{+h} \qquad A\,ch$

$\frac{1}{2} \qquad\qquad \frac{1}{2} \qquad P = 2^{-5}x^2y$

$B++ \qquad C++$

8.
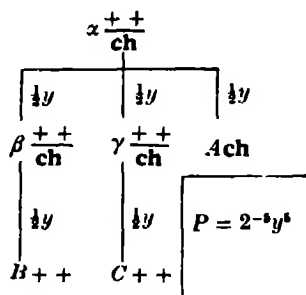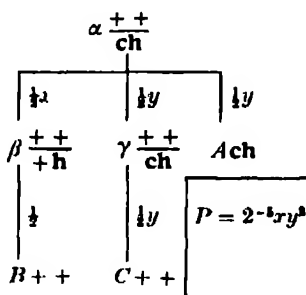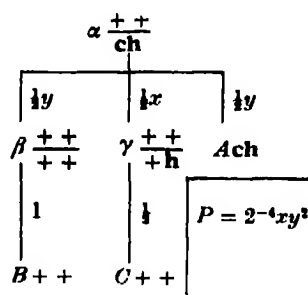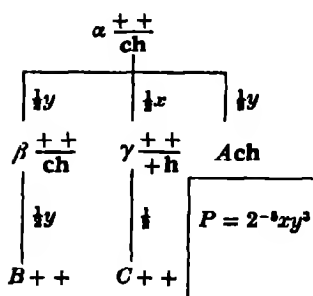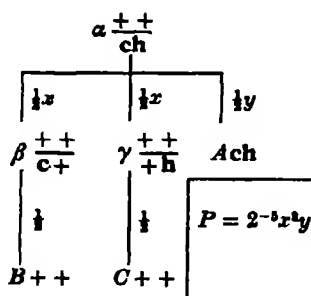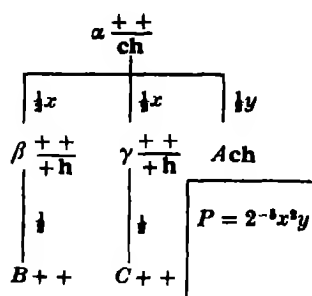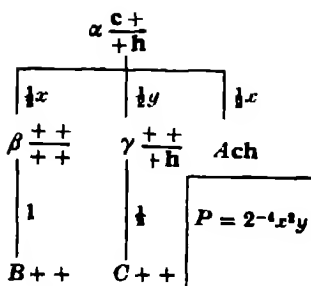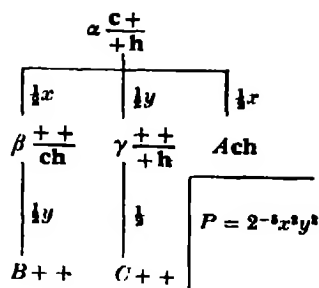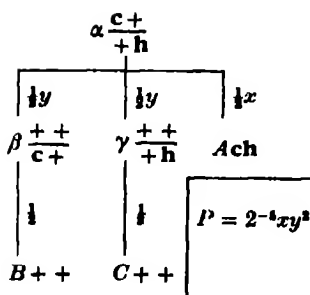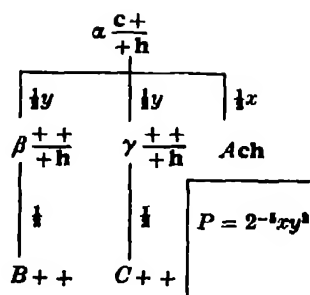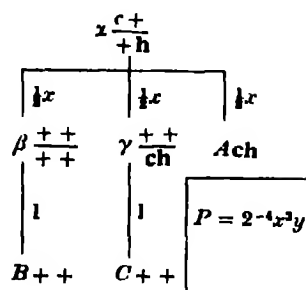
FIGURE 3. Sixteen possible explanations of the pedigree of figure 2. In each case the probability of the five different steps is given, together with their product. The overall probability is the sum of these products, multiplied by the probability that **c** and **h** should be compresent in α.

Fisher (1936) developed a most elegant method for the detection of partial sex-linkage in such cases. Suppose a sibship consists of N normal females, n normal males, A affected females, a affected males, and that

$$u = (N - n - 3A + 3a)^2 - (N + n + 9A + 9a),$$

then in the absence of partial sex-linkage the expected value of u is zero, in its presence it is $\frac{1}{2}k(1-2x)^2$, where $k = (N+n+9A+9a)^2 - (N+n+81A+81a)$, and x is the frequency of recombination. Thus if the sum of a large number of u values is significantly positive partial sex-linkage can be inferred, and its intensity estimated.

I have since shown (Haldane 1948) that if we calculate a polynomial for each sibship on the lines developed for the investigation of linkage between sex-linked genes, its logarithm can be expressed as a series in ascending powers of $(1-2x)^2$. The coefficient of $(1-2x)^2$ is Fisher's u. Thus the sum of Fisher's u scores gives a perfect test for the presence of linkage, though not a quite unbiased estimate of its intensity. There is, however, a further complication. In the absence of linkage the sampling distribution of Σu is not normal, but positively skew. So a high positive value gives a rather exaggerated estimate of the significance of the evidence for linkage. When allowance is made for this (Haldane 1946) most, but not quite all, of the data formerly regarded as significant remain so.

FIGURE 4. ♂, male homozygous for a partially sex-linked recessive. The sex of his maternal grand-parent is irrelevant.
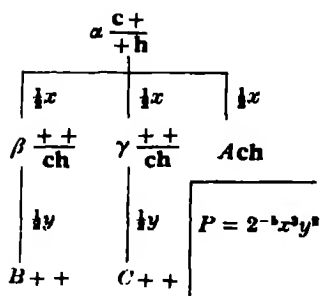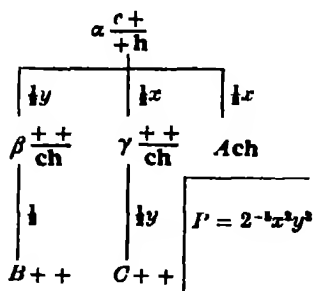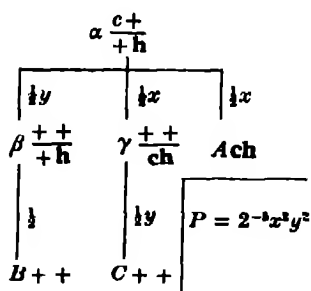
The Croonian Lecture was originally intended to be on 'local motion', and I shall therefore illustrate recessive partial sex-linkage by discussing spastic paraplegia, a disease in which the tonus of the limb muscles gradually increases until walking becomes impossible. Bell (1939) collected forty-four pedigrees in which one or more children of normal parents were affected with this disease.

Applying Fisher's method we have $\Sigma u = 1256 \pm 231$, and the estimated frequency of recombination is 17·5 %. The significance is not as high as it appears, since the sampling distribution in the absence of linkage is very skew positively. But it is not in doubt. It is wholly possible that while most of the families are segregating for a partially sex-linked gene, others are segregating for an autosomal one. To determine whether this is so, about five times the present number of families would be required, and it would be necessary to devise new statistical methods. There is also a suggestion, both from the results of the direct method applied to the progeny of cousin marriages, and the indirect method based on Fisher's 'u' scores, that a few cases diagnosed as spastic ataxia, and perhaps even as Friedreich's ataxia, may be due to partially sex-linked genes. The large majority are not (Haldane 1941 a).

On the basis of such statistical work I located seven genes on this segment. The standard errors of their distances are so large that I do not think that a map is worth publishing. However, the loci of dominant and recessive genes for retinitis pigmentosa, probably allelomorphs, lie about 30 units from the sex-determining or
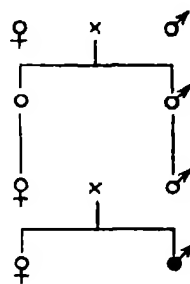
differential segment, while the loci of five other genes, namely those for achromatopsia, epidermolysis bullosa, xeroderma pigmentosum, spastic paraplegia, and Oguchi's disease, seem to lie between 21 and 44 units from it. In addition a lethal gene for convulsive seizures with mental deterioration is probably located in this segment (Snyder & Palmer 1943) while a gene concerned in some cases of hare-lip and cleft-palate may be so (Philip & Mather 1940)

The next step in this investigation will perhaps be the discovery of families in which colour-blindness is segregating along with a partially sex-linked gene such as those for spastic paraplegia or xeroderma pigmentosum In the latter case in particular we should be able to detect linkage in doubly heterozygous females between genes in the two segments of the X, and thus to produce a unified map Even more valuable would be the discovery of a gene as common as those for colour-blindness and anomalous matching, in either section of the X chromosome. The most hopeful field for such a discovery is among the antigens.

I have deliberately restricted my own work on human linkage to the sex chromosomes, because in every satisfactory pedigree sex as well as abnormality is recorded Other workers more industrious than myself have looked for linkage between autosomal genes. Since man has twenty-three pairs of autosomes, the probability that a particular pair of loci will lie in the same chromosome is of the order of $\frac{1}{23}$, though rather more, because the chromosomes are of unequal size On the other hand a recombination value of over 25 % is unlikely to be detected until data accumulate in very considerable quantity

Two genes are very possibly linked with those for the blood-group antigens These are the recessive gene for phenylketonuria (Penrose 1946a) and the partially dominant gene for allergy (Finney 1940, see also Finney 1941, 1942). A phenylketonuric apparently lacks some enzyme concerned in the metabolism of phenylalanine, and consequently excretes up to 1 g /day of phenylpyruvic acid As a further consequence there is a shortage of material for melanin formation, and the hair is of a lighter colour than that of other members of the family Far more important, thought is impossible Phenylketonurics are usually idiots or imbeciles, at best feeble-minded.

Penrose (1946a) has developed a statistical method for dealing with such cases, when the children in a family can be examined, but the parents cannot Consider a series of sibships in which some members are normal, others are phenylketonurics, some have the B antigen, i e. belong to group B or $A B$, others belong to other groups Consider what is to be expected if the loci concerned are on the same chromosome. In some sibships the recessive genes for phenylketonuria and absence of B antigen have been in the same chromosome in both parents In these, the sibs who are phenylketonurics will probably not possess antigen B, or more accurately, will be less likely than normals to possess it. If the genes are in different chromosomes in one or both parents, those who are phenylketonurics will probably possess antigen B Over a group of sibships there will be no association between the two characters, but in any particular sibship they will be positively or negatively associated

Now if we observe any pair of sibs, they must fall into one of the nine categories of table 6.

TABLE 6. THE NINE POSSIBLE TYPES OF SIB-PAIRS IN A SIBSHIP SEGREGATING FOR THE B ANTIGEN AND PHENYLKETONURIA. EXPLANATION IN TEXT

	BB	Bb	bb	total
NN	$a_{11}+$	$a_{12}-$	$a_{13}+$	c_1
NP	$a_{21}-$	$a_{22}+$	$a_{23}-$	c_2
PP	$a_{31}+$	$a_{32}-$	$a_{33}+$	c_3
total	b_1	b_2	b_3	1

Here N and P denote normality and phenylketonuria B and b the presence and absence of the B antigen. Thus a pair of sibs of whom one is normal and one phenylketonuric, but both belong to group O, falls into the category $NP.bb$. If the two gene pairs are located in different chromosomes the expected number E_{23} of sib-pairs in this category will be the product of the numbers of NP pairs and bb pairs, divided by the total, that is to say b_3c_2/s If they are in the same chromosome it will be less. In general, linkage will increase the numbers in the categories labelled $+$ in table 6, and diminish it in the remainder. Penrose (1946a) found the figures of table 7.

TABLE 7. NUMBERS OF SIB-PAIR TYPES FOUND BY PENROSE, WITH EXPECTATIONS IN THE ABSENCE OF LINKAGE

	BB	Bb	bb	total
NN	17 (9·125)	11 (16·947)	89 (90·928)	117
NP	10 (15 365)	34 (28·535)	152 (153·100)	197
PP	1 (3 510)	7 (6·518)	37 (34·972)	45
total	28	52	279	359

In this table the bracketed numbers are the expectations in the absence of linkage. Thus $9 \cdot 125 = \dfrac{117 \times 28}{359}$. In the absence of linkage, Penrose finds that

$$\xi = \left[\frac{a_{11}}{E_{11}} + \frac{a_{13}}{E_{13}} + \frac{a_{31}}{E_{31}} + \frac{a_{33}}{E_{33}} + \frac{4a_{22}}{E_{22}} - \frac{2a_{12}}{E_{12}} - \frac{2a_{21}}{E_{21}} - \frac{2a_{23}}{E_{23}} - \frac{2a_{32}}{E_{32}}\right]$$
$$- \left[s\left(\frac{1}{b_1} + \frac{4}{b_2} + \frac{1}{b_3}\right)\left(\frac{1}{c_1} + \frac{4}{c_2} + \frac{1}{c_3}\right)\right]^{\frac{1}{2}}$$

is normally distributed with mean zero and unit variance. A positive value indicates linkage. In this case the value is $+1\cdot 51$ which is not in itself significant. But Penrose informed me that when the O antigen and the two forms of the A antigen are also taken into consideration, the value rises to $1\cdot 78$. Since this is in the direction expected on theoretical grounds, the probability of obtaining so large a value by chance is $0\cdot 046$ or one in twenty-two. Such a value is usually taken as on the borderline of significance. A few more families may well establish this linkage conclusively.

Finney (1940) used modifications of Fisher's *u* statistics, and concluded from Zieve, Wiener & Fries' (1936) data that the gene for allergy was linked with those for the blood groups. However, it must be emphasized that the genetics of allergy are not so simple as those of phenylketonuria The probability of obtaining so large a deviation in the expected direction is 0·04, after correcting for skewness. His result and Penrose's are weaker evidence for linkage than appears at first sight because man has twenty-three pairs of autosomes, so the *a priori* probability of linkage is only about 0·04. This means that considerably stronger evidence for linkage is required than in *Drosophila melanogaster* where the *a priori* probability is about 0·5

Burks (1939) published preliminary results which very strongly suggest linkage between genes for hair colour and defective teeth Unfortunately only a statistical summary was given, and as I have pointed out elsewhere (Haldane 1941 *b*) other explanations besides linkage are possible, though perhaps not very likely She also obtained evidence of linkage between genes for myopia and eye colour Penrose (1935) and Rife (1941) obtained suggestions of linkage between blood groups and hair colour, and interdigital pattern and left-handedness respectively

Finally, Kloepfer (1946) has made a most comprehensive study involving nineteen characters, and obtained evidence suggesting a number of linkages The most impressive are those between eye colour and flare (or projection) of the ears, and between ability to taste phenyl-thio-urea and ear size. Unfortunately nothing is yet known as to the genetics of ear size and structure

Finally, there is massive negative evidence showing that various genes, notably those for blood groups, blood types (*M* and *N*), and ability to taste phenyl-thio-urea, are not linked with one another nor with sex Such work is inevitably tedious, but it is striking how long a time elapsed before linkage was discovered in poultry or peas, and how rapidly knowledge accumulated once the first linkages were discovered

Up till now we have considered the behaviour of genes in so far as they reproduce their like (or perhaps better, are copied exactly) at each nuclear division. When this does not happen, a new type of gene arises which generally, but by no means always, reduces the fitness of the organism either (*a*) at once, if it is a dominant, (*b*) when it appears in a male, if it is a sex-linked recessive, or (*c*) when two genes of the new type are contributed by different parents to the same zygote, if it is an autosomal recessive. This process of change is called mutation Clearly it may be due to a failure of the copying process, or to a change induced in the model between copyings by physical means such as X-rays or chemical means such as $\beta\beta'$-dichlorethyl disulphide. Mutation occurs spontaneously, that is to say under normal conditions, in all organisms so far studied; but as it is a rare process, it can only be measured when vast numbers are available The rate was first measured in *Drosophila melanogaster*, then in *Zea Mays*, and finally by Gunther & Penrose (1935) and Haldane (1935) in man. More exact estimates, fully confirming these figures, have been made in Denmark in the last 6 years.

The rate can be measured directly, as was done by Mørch (1941) for achondroplasia, or chondrodystrophy. This is the condition found in the familiar short-legged type

of dwarf. In 1938 there were eighty-six such dwarfs in Denmark among 3,800,000 people. They have a very low fertility, but when they breed, about half their offspring are similar dwarfs. The large majority of dwarfs, however, are the offspring of normal parents with no dwarfs in their families. It is clear that the gene for dwarfism arises sporadically by mutation Out of 132,761 children born of normal parents in hospitals in Copenhagen and Lund over a period of 21 years, eleven were dwarfs of this type. This gives a mutation rate of $4 \cdot 1 \times 10^{-5} \pm 1 \cdot 2 \times 10^{-5}$ per normal gene per generation, or about $1 \cdot 2 \times 10^{-6}$ per year, since the mean age of normal parents is 35 years. The probability that the true value should be as low as 10^{-5} per generation is $0 \cdot 0011$, the probability that it should be as high as 10^{-4} is $0 \cdot 0001$, so the order of magnitude is certain.

Mørch also estimated the mutation frequency indirectly. Most such dwarfs die at or within 2 days of birth, and a number more in the first year of life, but after this their expectation of life is normal. If 80 % die in the first year, which is his estimate, there would be 415 such dwarfs in Denmark but for this mortality, or a frequency of $1 \cdot 09 \times 10^{-4}$. Now 108 dwarfs had 27 children, and their 457 normal sibs had 582, thus their fitness from a Darwinian point of view is $\frac{27}{108} \times \frac{457}{582}$, or $f = 0 \cdot 204$. That is to say in each generation natural selection effectively eliminates 80 % of the dominant genes, and but for mutation there would be no dwarfs left on earth within seven generations, or say two centuries, if Danish figures are typical. However, the two processes are in approximate equilibrium. So if x is the frequency at birth, the mutation rate $\mu = \frac{1}{2}(1 - f)x = 4 \cdot 3 \times 10^{-5}$. The factor $\frac{1}{2}$ arises because we are dealing with a population of chromosomes equal to twice the population of human beings. The two estimates agree very well, but the second is much less accurate, since it depends on the figure for the infant mortality. Mørch, using a rather different argument, gets $4 \cdot 8 \times 10^{-5}$.

Professor Penrose has pointed out to me that Mørch's data are open to three criticisms. In some pedigrees, though not in any of those which he collected, there is evidence that the gene for achondroplasia can fail to manifest itself, as in Richsbieth's (1912) pedigree 608 The cases where two normal parents had more than one achondroplasic child may be due to this cause or to a mutation at an early stage in the development of a gonad. A correction for this possibility makes very little difference to the estimate of the mutation rate, since the gene is detected on its first appearance, even if this be occasionally delayed Secondly, Mørch did not personally examine all the infants, and it is possible that some may have been wrongly diagnosed This is plausible, since he himself failed to confirm the diagnosis of achondroplasia made by another worker in a Norwegian family. Finally, the frequency increases with parental age to an extent inexplicable if all the dwarfs born of normal parents are mutants, but explicable if some of them are due to bad prenatal conditions, as with mongoloid imbeciles. It may therefore well be that Mørch's figure is too high. But the true value is almost certainly above 10^{-5}.

For most diseases only the indirect method is available. Andreassen (1943) has applied it to haemophilia in Denmark. However, I believe (Haldane 1947b)

that his calculations give rather too low a result. Haemophilia is due to a sex-linked recessive gene. Hence only about one-third of the genes for haemophilia in a population are exposed to natural selection at any moment. More accurately, if μ and ν are the mutation rate in the female and male sexes respectively,

$$2\mu + \nu = (1 - f) x,$$

where x is the frequency in males at birth. Now there were just eighty-one haemophilics alive in Denmark in 1941, and their mean life is 18 years compared with 55 for Danes in general. So $x = 1.33 \times 10^{-4}$. The fitness f, that is to say the mean number of progeny, compared with that of the population in general, appears to be 0.28 I have criticized Andreassen's much higher figure. $f = 0.59$. It follows that $2\mu + \nu = 9.6 \times 10^{-4}$, or a mean mutation rate of 3.2×10^{-4}. Now if the mutation rates were equal in the two sexes, i e. $\mu = \nu$, nearly a third of all haemophilics would be single cases due to mutation in homozygous mothers Andreassen has shown that the gene for haemophilia is not completely recessive. Heterozygous women sometimes bleed abnormally, but always have an abnormally long coagulation time, by which they can be detected. Using this technique he has not yet found a case where the mother of a haemophilic was homozygous. Doubtless such a case will be found. But it can be concluded that ν is much larger than μ, very likely ten times as large If this is correct we should have, very roughly, $\nu = 8 \times 10^{-5}$, and $\mu = 8 \times 10^{-6}$

Similar estimates are available for three other conditions. Gunther & Penrose (1935) found $4–8 \times 10^{-6}$ for epiloia, Philip & Sorsby (unpublished) found 1.4×10^{-5} for retinoblastoma, and Møllenbach (according to Kemp 1944) finds $5–10 \times 10^{-6}$ for aniridia (Kemp's figure of double this value appears to be the mutation rate per zygote, not per gene) The median rate is about 10^{-5} Unfortunately, this method cannot be applied to autosomal recessive conditions.

Pätau & Nachtsheim (1946) have estimated the mutation rate of the autosomal dominant gene Pg which is responsible for the Pelger anomaly, a failure of segmentation of the nuclei of polymorphonuclear leucocytes. $Pg/+$ individuals are thought to be less resistant to disease than $+/+$, whilst by analogy with rabbits, it is suggested that Pg/Pg is a lethal genotype. However, too little is known as to the viability of heterozygotes to allow an indirect estimate of the mutation frequency. The authors estimated the frequency of the condition as 0.001, and found that out of twelve persons showing the Pelger anomaly, and both of whose parents could be examined, one parent was affected in ten cases, neither in two cases. This gives a mutation rate of $\frac{1}{2} \times 10^{-3} \times \frac{2}{12}$, or 8×10^{-5}. Even if the frequency were accurately known, and if 120 cases had been examined instead of twelve, this estimate would be somewhat high, simply because the cases with both parents living are a selected group, and include a higher fraction of cases with normal parents than of those with one affected However, the order of magnitude agrees well with the figures given above, and is unlikely to be incorrect by a power of ten

It is certain that these figures are not representative Consider a well-known and unmistakable dominant such as lobster claw. Five families with this gene are known

in England. They are quite fertile, but presumably their fitness, or net fertility, is a little below the average, or the condition would be commoner. Lewis & Embleton's (1908) pedigree goes back to a son of allegedly normal parents born in 1793. In the case of such a conspicuous abnormality mutation is a far likelier explanation than adultery. But it is extremely doubtful whether the mutation occurs in Britain once in 10 years. Five isolated cases were described in Britain between 1895 and 1918 That is to say its mutation frequency is of the order of 10^{-7} per generation. This is probably a much more representative figure than those of 10^{-5} or over. Unfortunately, the indirect method becomes quite unreliable when, as in this case, the fitness is near unity. Finally, we have such cases as that of the 'porcupine men' of the Lambert family (literature, see Cockayne 1933), a most striking dominant mutation, perhaps a translocation, carried by the Y chromosome. This has only been recorded once, and would have stood a good chance of being recorded in any civilized country, in the last 2000 years. It was twice described in the *Philosophical Transactions* of this Society. The mutation rate is probably below 10^{-10}.

A man or woman consists of about 2^{48} cells, that is to say a representative cell is separated from the fertilized ovum by about fifty mitoses. The primordial ova are all formed at birth, and do not undergo further mitoses A man may produce 2^{40} spermatozoa in a lifetime, so the mean number of mitoses is somewhat greater in the male than the female germ-line, but probably not over 100 in the former.

Thus a mutation rate even of the order of 10^{-4} means that the gene-copying process, at worst, goes wrong about once in a million times, whether as the result of a failure of copying, or of a change in between two copying processes A similar degree of accuracy in crystal growth would give a crystal with under ten flaws per millimetre, and 10^{10} successive flawless layers would give a perfect crystal several metres in length The living substance of our bodies is clearly far more accurately copied than the successive layers of a crystal.

In *Drosophila* the natural mutation rate is of the order of 10^{-6} to 10^{-5} per generation for the more mutable loci, such as that whose mutation produces a white eye, and considerably lower for the stabler genes. Natural mutation is increased about threefold by a rise of 10° C, and is therefore largely due to a chemical reaction As a generation in *Drosophila melanogaster* takes about 10 days and a fly contains about 2^{23} cells, while the mutation rate of the more labile genes is about one-fifth of that of man per generation, it follows that human mutation rates are about twice those of *Drosophila* per nuclear division, and about one two-hundredth of those of *Drosophila* per day, though the body temperature is about 13° higher. It has been calculated that natural radiations and particles of high energy will account for only 0·001 of the mutations in *Drosophila*. It is clear that if so they may account for about a fifth of those in man, and in view of the uncertainty of our knowledge as to the efficiency of particles from K^{40} and cosmic rays in producing mutations, and the different radiosensitivity of different genes, it is quite possible that radiation may account for most human mutation. Mørch found that the rate of mutation to achondroplasia increased with age, but it was not clear whether maternal or paternal age was most important. If this finding is confirmed it suggests a cumulative effect,

either of radiation, or of successive nuclear divisions, during a lifetime. The apparently higher rate in males suggests that the number of nuclear divisions may be an important factor in human mutation. To sum up, there are three possible known causes of mutation, a chemical reaction with a temperature coefficient, radiation, and imperfections of copying, which might have a positive or negative temperature coefficient. The first predominates in *Drosophila*, the second or third probably does so in man. There must be about a thousand achondroplasic dwarfs in Britain. If the ages of their parents at their births were determined, it would be possible to decide between these alternatives, since the egg of a woman of 45 has undergone no more nuclear divisions than that of a girl of 15. It is worth remarking that it is quite practicable to obtain data of this kind on populations of 40 million human beings, and wholly impracticable to do so on 40 million of any other mammal.

The mutation rate is probably more or less adaptive. Too high a mutation rate would flood a species with undesirable mutations, too low a one would probably slow down evolution. Man and *Drosophila melanogaster* have about the same rate per generation, and this could not be increased ten times without a very great loss of fitness (Haldane 1937). Other species such as five species of *Sciara* (Metz 1938) have far lower rates per generation though not necessarily less than the human rate per day. But it is doubtful whether the human rate could be lowered much further, since a substantial fraction of it is due to natural radiation. In fact a very great prolongation of human life, or at any rate of the reproductive period, might be incompatible with the survival of the human species.

I hope that, in this brief survey, I have shown that human genetics has reached the stage when it can claim to be a branch of biology with its own peculiar problems and methods. I have only dealt with a few of them. This lecture could equally well have been devoted to the human antigenic structure, to human prenatal physiology, or to variation in human sensory and intellectual capacity, all of which a human geneticist must study. If I have confined myself to the more quantitative aspects, my excuse must be that in dealing with a branch of science where erroneous views may have important political consequences, in such a lecture as this it is desirable to concentrate on those problems where political or social bias is least likely to be effective, and where we may hope to raise a solid theoretical structure by methods like those which have been fruitful in the other branches of science.

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A discussion on
the physiology of resistance to disease in plants

PHYSIOLOGY OF THE FACULTATIVE TYPE OF PARASITE

BY W. BROWN, F.R S.

HOST RESISTANCE TO FUNGI, CHIEFLY IN RELATION TO OBLIGATE PARASITES

BY F. T. BROOKS, F.R.S.

SOME EFFECTS OF HOST-PLANT PHYSIOLOGY ON RESISTANCE TO VIRUSES

BY F. C. BAWDEN

Professor W. Brown opened the discussion by giving a classification of plant diseases on the basis of the causal agent. This was for the purpose of pointing out certain broad differences between diseases of plants and of animals and of indicating which parts of the subject would be dealt with by the three speakers

PHYSIOLOGY OF THE FACULTATIVE TYPE OF PARASITE
BY W. BROWN, F.R.S.

In studies of plant disease it is customary to draw a sharp contrast between the facultative and the obligate type of parasite, and one can readily indicate some half a dozen points of difference. Thus the facultative parasite is one which typically kills the host cells and then lives on the dead remains, in contrast to the obligate parasite which, when invading a suitable host, lives for some time in a symbiotic state with the latter The obligate parasite may, as will be shown more fully by a later speaker, exert a localized killing action on the tissues of certain hosts and thereby bring about its own inhibition. The basis of this difference is considered to be that a facultative parasite is cultivable on a dead medium, that it is in reality a saprophytic fungus which has parasitic potentialities, whereas the obligate type of parasite cannot grow apart from the living cells of a suitable host. More guardedly one should say that the secret of cultivating the obligate parasite *in vitro* has not yet been discovered, but the time may come when this basis of distinction no longer holds.

Certain other features of difference are correlated with the foregoing, or at least are considered to be so. The facultative parasite is able to thrive on a variety of media and thus tends to be somewhat generalized in its parasitic behaviour, in contrast to the obligate type which shows a greater or less, and sometimes a remarkably fine, degree of specialization, which one ascribes to a fine discrimination in its nutritional requirements. As an outward manifestation of this nutritional

difference, the hyphae of the facultative parasite grow indiscriminately through the host tissue, i.e. both intra- and inter-cellularly, whereas mycelium of the obligate type is characterized by the presence of well-marked haustoria. These alone enter the living cells, or in the case of lower members of this group, the whole thallus of the parasite is intra-cellular.

A further broad distinction is referable to the time relationships shown in the process of attack and possibly also to the action of growth hormones. The host tissue is killed more or less rapidly by the facultative type of parasite and thus does not in general respond by cell proliferation. On the other hand, the symbiotic phase which is characteristic of invasion by the obligate parasite allows of this and so one finds in this case that hypertrophies (galls) of various kinds are frequently produced.

While the above comparisons are of general application, it should be remembered that there are exceptions to each one, and that there are intergrades with respect to all the characters stated. In particular there are obligate parasites which are not so highly specialized and facultative parasites which show a narrow range of selection. For this reason one may indulge the hope that a physiological analysis of facultative parasitism may lead in time to a better understanding of the more subtle problem presented by the obligate parasite.

I propose to deal with the physiology of the facultative parasite, but some of the conclusions will apply equally well to the other type. Parasites in the facultative group are *Botrytis cinerea*, *Rhizoctonia solani*, *Pythium de Baryanum*, and soft-rotting bacteria such as *Bacterium carotovorum* These all have the experimental advantages that they attack many common plants and that they can readily be grown on a variety of natural and synthetic culture media Their biochemistry is therefore open to study. I propose to outline what we know of the aggressive mechanism of such parasites and at the same time to indicate what means of resistance the plant is able to oppose to such action. For convenience in description I shall deal with the story in three phases (i) Pre-penetration phase; (ii) Process of penetration, (iii) Post-penetration phase.

(i) *Pre-penetration phase*

For the fungal spore lying on the surface of a plant a prerequisite to penetration is that germination should take place, and for this purpose certain environmental conditions must apply. These are, presence of oxygen, of moisture and temperature within a certain range. At this point it will be appropriate to point out what is a main difference in degree between the establishment of disease in plants and in animals, more particularly in warm-blooded animals. With the latter the environmental conditions prevailing at foci of infection are comparatively uniform, so that they do not in general exercise any control on the sequence of events. In plant disease it is otherwise, and one can definitely say that the environment is in most cases the all-controlling factor. If conditions are unsuitable, disease is checked at some stage or other; if they are suitable and remain so for a sufficiently long time

the disease becomes established and increases in a kind of geometrical progression. In other words, the disease takes on the epidemic form, as in the well-known example of potato blight.

Oxygen is practically always available to the germinating spore and calls for no further mention. Beyond stating that the temperature at which most phyto-pathogenic fungi germinate normally must be within the range 5 to 25° C, nothing further need be said of temperature at this stage. Moisture is the important controlling factor and in nature this is supplied as rain or as dew, though some fungi will germinate in the absence of free water, viz. in an atmosphere of sufficiently high humidity (90 to 100 % of saturation). Furthermore, adequately moist conditions must last for a sufficiently long time, as it appears to be a general rule that, whereas the ungerminated spore can withstand repeated wettings and dryings without damage, the germ-tube is destroyed by desiccation. Hence moist conditions must prevail until the fungus has established itself within the tissues of the plant, after which it is less exposed to the influence of drying. It is known that one day of wet weather is insufficient to enable the fungus of potato blight to establish itself, even at the most favourable time of the year, a succession of about three such days is necessary for the purpose. Similarly, the length of the dew period is of critical importance for the development of some diseases (e.g. powdery mildews), and it is noteworthy that with these the spores germinate and enter the host tissue with great rapidity.

It is in connexion with the necessity of moisture for spore germination that one notes a feature which is shown by some plants and which, though not a type of resistance in the true sense because the plants may in fact be highly susceptible, has nevertheless the effect of considerably reducing the chances of the plants being attacked. This is the presence of a more or less unwettable surface to the plant, arising from a waxy bloom on the cuticular layer or from an arrangement of epidermal hairs. This effect may be reinforced by the vertical orientation of the plant surface, with the result that drops of water are thrown off, or do not really come in contact with the surface, and so one of the primary conditions for the initiation of attack is not realized. This is one form of 'disease-escape' of which other examples could be quoted.

Granted that environmental conditions are suitable, the capacity of a spore to germinate is in the first instance inherent in itself. Spores of some fungi are unable to germinate in pure water, those of others do so. The capacity of any particular sample of spores to germinate on their own resources is influenced by such factors as their age and the conditions under which they were developed. Germinative vigour passes through an optimum with time, after which it gradually falls off to zero. Similarly, spores show reduced germinative capacity when derived from cultures which have been maintained at supra-optimal temperatures. As increased vigour of germination leads to a greater chance of successful parasitic invasion, it is obvious that the amount of germination shown by the inoculum under standard conditions is a factor of importance in the analysis of parasitic capacity.

There is another factor which may play a part at this stage and this arises from the host tissue. While there is no evidence that, in the normal way, the fungus exerts any influence on the host tissue previous to penetration, it has been clearly shown that substances diffusing in a purely passive manner from living cells of the host considerably affect the germination of the fungus. Often the effect is stimulative, but in others repressive. The permeability of the superficial host cells and the nature of the substances which diffuse out into water in contact with the plant surface (into the so-called 'infection drop') exercise by this means a controlling effect on the earliest stages of parasitic attack.

Antagonism between micro-organisms is known to play a part in determining the vigour of attack, both at the pre-penetration and later stages. In general, the effect when a parasite and saprophyte or two parasites are present in the same inoculum is a diminution of invasive capacity, though examples of the opposite kind (synergism) have been recorded. The mutual action appears to be based upon an accumulation of metabolites and not on competition for food material. This is a plant pathological illustration of the antibiotic phenomenon which is also well shown where a wound, which would if freshly made be readily invaded by a specific parasite, is protected from invasion if already occupied by saprophytes. Antibiotic effects are shown best of all in connexion with certain soil-borne parasites, for it has been repeatedly shown that the amount of damage caused by particular root-infesting fungi is greatest when other micro-organisms are excluded.

(ii) *Process of penetration*

Rhizoctonia solani, Pythium de Baryanum, Botrytis cinerea, and many others, both facultative and obligate, enter their host plants by penetrating directly through the cuticularized epidermis and the process has been followed in detail for a number of these fungi. Certain early experimenters claimed that the stimulus to penetration was of the nature of a tropism to substances diffusing out from the host tissue. It was even suggested that the presence or absence of such substances, or of a particular substance in the case of a highly specialized parasite, determined whether a fungus would enter or not. This chemotropic theory has now largely been abandoned, as more recent work has shown that fungal hyphae will penetrate membranes, natural or artificial, under conditions which rule out the possibility of such an attracting chemical factor. Thus the germ tubes of *B. cinerea* are able to penetrate a plant epidermis equally well from the inside as from the outside, i.e. away from the supposedly attractive substance as freely as towards it. It has also been suggested that the movement leading to penetration is the result of a tropism away from the metabolites of the fungus itself, but a little consideration of the gradients of such substances within the infection drop will show that this view also is untenable. In the circumstances the only available alternative is that the stimulus to penetration arises from mechanical contact, is a haptotropism, of which there are other well-defined examples in the plant kingdom.

The mechanism of the act of penetration is also undoubtedly mechanical. The evidence for this conclusion is partly that fungi are not known to be able to secrete a substance capable of dissolving or softening the outer cuticularized layer of plants, and partly that the visible details of the process of penetration suggest that it is a mechanical act. The germ-tube of *B. cinerea*, growing along the surface of the plant, becomes attached to the latter in the region of its tip; growth in length ceases temporarily and the fungus attempts to penetrate at the region of attachment ('appressorium'). That the process of attachment is stimulated in a mechanical way is shown by the fact that appressoria are freely formed on a glass surface. The purpose of these structures is to take up the back-thrust arising from mechanical penetration. The growth which penetrates the cuticle is extremely fine and it is only after the cuticle has been passed that the hypha swells out to its normal dimensions.

The different penetrative capacities of fungi and the capacity of plant surfaces to resist penetration by a fine point are subjects which call for examination in this connexion. By the use of formalized gelatine membranes of graded hardness it has been shown that the series *B. cinerea*, *Penicillium glaucum*, *Rhizopus nigricans* is one of diminishing penetrative power, so that it is only the least-protected structures (e.g. certain soft fruits) which the last-named is able to penetrate. As regards cuticular resistance, it is known that young leaves in which the cuticle is still thin are more readily penetrated than mature ones. This applies, for example, to the penetration of apple leaves by the scab fungus. It is also known that part of the resistance offered by the cuticle depends upon the hydrostatic pressure of the underlying epidermal cells, for if this is removed by plasmolysis, or in the extreme case by killing, entrance is much easier. This partly explains why leaves are more readily entered in the senescent stage than when in their full vigour, the other factor concerned being the greater exosmosis of food materials to the exterior which enhances the penetrative power of the fungus.

The cuticle (and suberized bark) is the first line of resistance to fungal attack and is the only line of resistance for many plants against specific fungi. Hence the type of parasite ('wound parasite') which gains entrance through wounds. These may be large and obvious as with broken branches or split fruits, but may be minute as when they are caused by the stylets of aphides or by normal processes of the plant, e.g. where a leaf has been recently shed or where a side root emerges from a larger root.

Many parasitic fungi circumvent the cuticular barrier by entering through stomata or lenticels. This method is the one generally adopted by plant-pathogenic bacteria so far as these affect overground parts. Whether or not germ tubes are attracted to stomata chemotropically is a debated point. Finally, some parasites gain entry through weakly protected structures (e.g. floral parts, in particular the stigmatic surface) or through moribund or dead tissues. With many herbaceous plants there is a considerable amount of dead tissue present by the time seed is matured, and this feature lays the plant wide open to the attack of certain fungi.

Hence the general experience that seed production of many flowers and vegetables is practicable only in certain areas where the climate is particularly inimical to fungal attack.

(iii) *Post-penetration phase*

The outstanding feature shown when a fungus such as *Botrytis cinerea* enters the tissue of a susceptible plant is that the cells of the latter are disorganized some distance ahead of the position occupied by the invading hyphae. Disorganization takes the form of killing of the living protoplasts and solution of certain cell-wall constituents, mainly pectic in nature, with the result that coherence of the tissue is lost, i e. the tissue is rotted   Extracts are readily prepared from the hyphae of *Botrytis* and similar fungi and from bacteria and these reproduce the two-fold action on a large range of plant structures. In extracts of *Botrytis* the significant substance present is a pectinase enzyme and it has not been found possible so far to separate a cell-killing from a cell-wall dissolving substance. Provisionally one must assume that the enzyme acts as a cytolytic toxin. Though pectinase enzyme is the most prominent agent of attack on tissue, it would not be safe to assume that other metabolites play no part. It is known, in fact, that with a few fungi, e g. *Sclerotium Rolfsii*, oxalic acid is excreted in a concentration sufficient to facilitate parasitism.

The mechanism of attack envisaged is therefore as follows. The fungus continues to advance through the tissues, following behind a screen of cells which have been killed and softened by pectinase enzyme which is excreted by the hyphae and which diffuses ahead of the latter. Parasitic invasion will continue indefinitely, provided nothing occurs to interfere with the process. It remains to consider the nature of these interfering factors.

There is considerable scope for experiment at this stage. The method is simply to introduce a variety of fungi or bacteria into a range of host tissues and to determine whether, and if so, how soon and by what means, invasion is arrested. The behaviour of the ineffective fungus is obviously of as great interest as that of an actual parasite. Numerous specific problems of this nature can be stated, and it would seem that the difficulties which stand in the way of obtaining a fairly clear picture are largely those of biochemical technique. In particular, certain intricacies of enzymic behaviour call for careful examination. A number of types of resistance have been recognized, and these will now be illustrated.

Internal resistance may arise from the presence in the plant of substances which are antagonistic to the growth of fungi or of most fungi   Substances of this nature are acids, tannins, ethereal oils, glucosides, etc. Fungi which attack highly acid structures (e.g. young apple fruits, citrus fruits) must obviously be able to grow in distinctly acid media, and one can readily confirm that parasites (e.g. species of *Sclerotinia, Penicillium*) of such tissues have in fact a high acid tolerance. Similarly, fungi which invade onion tissue are relatively insensitive to the oil present there. Factors of this nature have the effect of severely limiting the number of fungi which could begin to attack the tissues concerned, it does not follow, however, that

any fungus with a sufficient degree of tolerance would necessarily act as a parasite, for there are further obstacles to invasion as will appear below.

A less obvious type of resistance, which also depends on the composition of the plant sap, occurs where there is nothing of an inhibitory nature present in the sap as such, but inhibition of growth is quickly brought about by the metabolism of the micro-organism itself. This effect is well shown in the failure of some bacterial species or strains to attack certain vegetable tissues. The reaction of the sap in the neighbourhood of the inoculum rapidly moves to an acid point and this leads to stoppage of attack. A study of the biochemistry of acid-production by these bacteria is indicated.

Inhibition of the parasite, after a certain amount of growth, by its own staling products probably accounts for the limitation in size of the lesions caused by many 'spotting' fungi (e g. *Gloeosporium*, *Phyllosticta*). In other cases the factor which arrests development is probably desiccation by the environment. The capacity of facultative parasites to kill, to a greater or less extent, the cells of the host in advance of the growing hyphae is an obvious means of progression but it brings the attendant danger that the lesion becomes readily dried, especially when the part attacked is a thin structure like a leaf

Defensive reactions stimulated in the host tissue by presence of a parasite may be mechanical or chemical. The former will be dealt with by a later speaker. To what extent, and even whether at all, plant tissues develop specific chemical means of defence against fungal and bacterial invaders is a matter which is still disputed. It is certain that no antigen-antibody relationship has been demonstrated in fungal and bacterial diseases of plants comparable to what is known to occur in animal pathology. Claims for an acquired immunity to particular cryptogamic parasites have been advanced by some workers but these are not generally accepted. Some of the findings are disputed, in others the effects claimed are rather insignificant, being confined to the neighbourhood of the lesion so that the inhibitory effect is as likely to have arisen from metabolites of the parasite as from a response of the host Some importance has been attached in this connexion to the frequent occurrence of substances of a phenolic nature in the neighbourhood of fungal lesions but there is no clear proof that these do in fact contribute materially to stopping invasion.

It may be suggested that much of the work which has aimed at elucidating the basis of internal resistance to disease in plants has taken too narrow a view of the problem. The search has been for substances which are inhibitory or retardatory to the *growth* of the parasite, and there is no doubt that a number of satisfying explanations have emerged. Even if such instances are numerous, they still fall very far short of the general problem, which is to explain why any plant is inherently resistant to the vast majority of fungi which have parasitic tendencies Furthermore, there is virtually an unlimited number of problems of the type represented in the following scheme:

	plant *A*	plant *B*
fungus α	+	−
fungus β	−	+

Fungus α attacks plant A but not B, and conversely for β. Plant B may have some feature, such as the presence of highly acid cell-sap or of inhibitory oil, which adequately explains the failure of fungus α to attack, whereas plant A is attacked. But the difficulty then arises as to how one is going to explain the converse behaviour of fungus β. It is most unlikely that a simple hypothesis of the presence or absence of growth-affecting substances will suffice to deal with the multiplicity of cases which can be put forward.

Growth of the parasite in presence of the host tissue is merely one of the requisites for establishment of parasitism. It is also necessary that the parasite should possess an attacking mechanism and that this should continue to act. At present we know that a significant part at least of this mechanism is the tissue-destroying enzyme pectinase. It is important therefore to examine this enzymatic action and to determine whether there are conditions under which it fails to work. Study of this problem has not advanced very far as yet, but enough is known to enable one to separate out a number of subsidiary problems.

Within limits a correlation has been detected between parasitic vigour and the capacity to excrete pectinase enzyme into culture media. This is shown, for example, by various strains of vegetable-rotting bacteria. The following is a further example. *Botrytis allii* is inactive when introduced into the flesh of apple fruit but causes appreciable attack if a trace of suitable nitrogenous compound is added with the inoculum. This fungus grows readily on apple-fruit extract without producing detectable pectinase, but if the extract is reinforced with nitrogenous compound (e.g. asparagin, KNO_3) the enzyme is freely produced. .

Nevertheless, this correlation is not general, as has been known for a long time and one's recent experience has abundantly confirmed. A comparison of the fungi *B. cinerea* and *Pythium de Baryanum* is interesting in this connexion.

On a wide range of cultural media, including decoctions of potato of various strengths, *Botrytis cinerea* readily produces active enzymic solutions. *Pythium de Baryanum* on ordinary potato decoctions and on most artificial media shows a negligible amount of enzyme secretion. Nevertheless, *P de Baryanum* is parasitic on ordinary mature potato tubers and *Botrytis cinerea* is not The fact that the former does attack involves no contradiction, for, in spite of its feeble capacity to produce the enzyme on various decoctions, strong enzymic preparations are readily made by extracting potato tissue which has been parasitized by the fungus. It is clear therefore that enzyme production depends not on the fungus only but also on the medium on which it is grown, and that some fungi are more selective than others in the latter respect. As for the failure of *B. cinerea* to attack potato tubers, this cannot be set down to any anti-effect of potato sap, for *Botrytis* spores develop freely on the cut surface of potato tissue and potato media of all kinds are highly suitable for the culture of this fungus.

There is an interesting difference between enzymic preparations of *B. cinerea* and *Pythium de Baryanum* when tested on living potato tissue. The method of determining the activity of pectinase solutions is to note the time required for loss of

coherence in microtomed disks of uniform thickness when completely immersed in the active liquids. When the test is made by placing a small quantity of the enzymic solution on the surface of a bulk of potato tissue, the amount of rotting brought about by *Botrytis* enzyme is much less than by an equal amount of *Pythium* enzyme of the same strength as determined by the standard method. This points a way to an understanding of the failure of *Botrytis* to attack potato tubers. When an inoculum of *Botrytis* spores is placed on the cut surface of potato tuber, a small amount of enzyme is excreted during early growth but this is ineffective, and is in fact deactivated.

The mechanism of this deactivation is not yet clear but there is evidence that it is modifiable by a number of simple treatments. Thus when potato tissue is raised to a state of complete turgor, which typically involves an increase of its water-content by about 5%, its susceptibility to inocula of *Botrytis* spores and to small quantities of the fungal enzyme is distinctly increased. A similar sensitizing of the tissue, both to fungus and to fungal enzyme, is produced by holding the tubers for some days at 30 to 35° C, an effect which is partly at least reversible, as the tissue regains its resistance when kept for some time at lower temperature. Similar results have been obtained with other fungi and other host tissues. The physiological analysis of these effects should lead to a better understanding of a type of resistance which seems to be based upon an anti-enzymic and not upon an anti-growth property of certain plant tissues in their normal condition

There are further differences between the pectinase enzymes of *Botrytis* and *Pythium*, for instance as regards optimal pH, sensitiveness to various salts, and resistance to desiccation. The interpretation of these differences may be that the enzymes are different in themselves or that they are the same but that some of their properties are conditioned by other metabolites produced by the particular fungi. It is clear that detailed enzymological study is called for in this connexion.

The parenchyma of many plants is quickly disorganized when treated with an excess of *Botrytis* pectinase enzyme, but many of these plants are resistant to the fungus itself, even when the latter is introduced into the tissue. Reasons for this limitation of the fungus have been given above. There remains another type of resistance, viz. that shown by the leaves of mosses and thalli of hepatics. These are sensitive neither to the fungus nor to its enzyme. As all the available evidence is to the effect that the protoplast-destroying constituent of the fungal extract functions only after action by the wall-dissolving substance (if in fact the two substances are not the same), immunity in this case ultimately rests upon the composition of the cell-walls. To interpret this type of resistance a better understanding of the chemistry of cellulose, hemicellulose, pectin, and such like substances is needed.

HOST RESISTANCE TO FUNGI, CHIEFLY IN RELATION TO OBLIGATE PARASITES

BY F. T. BROOKS, F.R.S.

The factors in the host conferring resistance to invasion by fungi which are obligate parasites are generally much more difficult to elucidate than where facultative parasites are involved, for the latter can be grown on culture media and their enzyme systems and other biochemical properties can be investigated in detail. At present, apart from the early stages after spore germination, a rust fungus can only be studied in relation to its host, and a clear analysis of the physiology of parasite and host respectively has not yet been achieved.

Even in the rusts, however, structural features of the host occasionally confer some degree of resistance. In the wheat variety, Webster, profuse development of sclerenchyma in the stem checks mycelial growth of *Puccinia graminis* and confers a measure of resistance (Stakman, Levine & Griffee 1925), although other factors are also involved such as the tough character of the epidermis which impedes exposure of the pustules. Young barberry leaves are readily penetrated by the sporidial germ-tubes of *P. graminis*, whereas old leaves, provided with a thicker cuticle, are not infected. There is evidence, too, that the relative thickness of the cuticle is a factor in determining whether powdery mildews cause infection (Corner 1935). In connexion with attack by *P graminis* on wheat it has been suggested (Hart 1929) that in some varieties tardy opening of the stomata in the morning confers a kind of functional resistance because the delicate germ-tubes of the spores on the surface are desiccated before penetration can be achieved. There is, however, some uncertainty about this interpretation.

In most examples of resistance to rust attack obvious structural features do not come into play, and the relationship of host and parasite is essentially bound up with subtle protoplasmic properties of the two organisms. The first stage in the elucidation of this relationship was described some years ago with regard to infection of wheat by *P. glumarum* (Marryat 1907) In an extremely resistant variety the only external sign of attack is the presence of minute necrotic flecks on the leaves, contrasting with abundant spore pustules on the otherwise normal leaves of the fully susceptible variety. Microscopic examination shows that the rust fungus enters the stomata of the resistant variety but sets up a violent disturbance as soon as it comes into contact with the mesophyll cells. This results in their speedy death and also in the death of the mycelium, which only continues to thrive in contact with living host protoplasm In the fully susceptible variety the rust establishes a kind of common life with its host, and there is no evidence of antagonism between the two organisms. Intergrades between the two types of reaction occur. Similar phenomena were subsequently described in varieties of wheat highly resistant to *P. graminis* (Allen 1923). Resistant plants which behave in this manner in response to parasitic invasion are said to be 'hypersensitive' to attack (Stakman 1915). Thus we have the paradoxical situation in which resistance seems to be bound up with

a lethal attack by the parasite. At present we can do little more than speculate about the physiological relations of host and parasite in such reactions. The death of the host cells and the death of the fungus must both be taken into account. In the attack on the resistant host the mesophyll cells are adversely affected even before the death of the hyphae, so it seems likely that some substance is secreted by the fungus which initiates the disturbance. The host cells in turn may secrete other substances lethal to the parasite, although the death of the latter may perhaps be due to starvation. The former supposition is the more likely because of the rapid deleterious influence on the parasite. In this connexion there is some evidence that wheat leaves affected by *P. triticina* or *P. glumarum* contain toxins which depress the germination of spores of these species, the effect being specific for each rust (Parker-Rhodes 1939). In semi-resistant varieties the struggle between host and parasite is prolonged; a few cells may be killed, but the fungus continues to live although impeded in its growth. In the susceptible host there is harmony with the parasite and abundant haustoria are present; any substances secreted by the fungus do not have an adverse effect, perhaps on account of inadequate concentration or because they are neutralized by the host protoplasm. It is claimed that in susceptible and resistant reactions to specific rusts the host cells become more and less permeable respectively (Thatcher 1943), but it is not known precisely how these changes are induced. In relation to rust fungi numerous attempts have been made to account for the differences between resistant and susceptible host varieties in biochemical terms, but so far without any satisfying result.

Hypersensitiveness has also been described in relation to attack by other kinds of fungi, notably in certain oat varieties resistant to *Ustilago avenae* and *U. kolleri* (Western 1936).

In the type of resistance indicated by hypersensitiveness the factors conferring resistance must be of a subtle kind because changes in environment (which affect both host and parasite), nutritional alterations of the host (which also influence the fungus), and even the presence of another parasite in the tissues may all change a resistant reaction to a susceptible one in a given variety. As regards the last consideration it has been shown that certain extracts from bunted plants of Little Joss wheat are less toxic to spores of *Puccinia triticina* than are extracts from healthy plants (Parker-Rhodes 1939). Similarly, a susceptible variety may become resistant under certain conditions.

In some kinds of host closely related to others which exhibit resistant or susceptible reactions to rust fungi there is no apparent reaction, for the inoculated plant remains perfectly normal as seen by the naked eye. Entry occurs through the stomata in the usual way, but the fungus soon dies either without exercising any appreciable lethal influence or occasionally after killing the guard cells between which it entered. Such hosts appear to be entirely inappropriate for the life of the fungus, but further examination of this type of behaviour is required. This state of affairs is closely similar to the penetration by rust fungi of plants which are quite unrelated to their proper hosts.

Some varieties of wheat which are susceptible to *P. graminis* in the seedling stage become resistant as they approach maturity. This is known as mature-plant resistance and is of great importance in breeding varieties capable of withstanding attack under field conditions. This character is inherited in a simple way, but the qualities in the host conferring this type of resistance are virtually unknown. Perhaps changes in host metabolism account for this difference; it has also been suggested that tardier opening of the stomata as the plant reaches maturity invokes the kind of functional resistance already referred to, although this explanation is not wholly applicable (Goulden, Newton & Brown 1930).

There are nearly 200 physiologic races of *P. graminis* affecting wheat, which produce diverse types of resistant and susceptible reactions in a range of varieties. There is no doubt about the differences between these races, but, taking such a multitude into account, the complexity of the problem of interpreting resistance to this fungus is fully revealed.

Erysiphe graminis, the powdery mildew of cereals and grasses, is a totally different kind of obligate parasite which infects only the epidermal cells of the host, invariably by cuticular penetration. Initially the process of entry is essentially the same as penetration by the germ-tubes of *Botrytis cinerea*, but growth in the host never proceeds beyond the formation of haustoria in the epidermal cells. The cuticle is penetrated in a purely mechanical way after which an enzyme system comes into play that changes the cellulose layer below into a papilla from which the haustorium emerges. In the immune wheat variety, Persian Black, the initial stages of penetration are the same up to the time of formation of a papilla, but then the process stops, the host cell remaining uninjured (Corner 1935) It is suggested that toxins in the host cell prevent the emergence of a haustorium; certainly the penetrating germ-tube quickly dies. In plants unrelated to the Gramineae *Erysiphe graminis* may also initiate infection but the process never gets beyond the papilla stage, and when the cuticle is thick the cellulose layer below remains unaltered and no papilla is formed. With another mildew, *E. polgoni*, a different kind of reaction is exhibited by resistant varieties of red clover (Smith 1938). Here there is sharp antagonism between the host cell and the fungus penetrating it, both being killed; sometimes a few contiguous cells are also killed.

With *Synchytrium endobioticum*, the cause of wart disease of potatoes, other phenomena are associated with invasion. In a susceptible variety the protoplasm of the fungus spore passes bodily into an epidermal cell and grows until it reaches the reproductive stage; at the same time the host cell enlarges and the neighbouring cells are stimulated to divide actively so that with successive infections large galls or warts are formed (Curtis 1921). One supposes here that some substance is produced by the parasite or by its action on the host protoplasm, which on diffusion into contiguous cells causes them to divide. In an immune variety the parasite also invades the surface cells, but quickly dies without entering on the reproductive stage and without stimulating the host cells to divide; in some immune varieties there is no adverse effect on the host cells (Cartwright 1926), but in others the invaded cell

and a few adjacent ones are killed (Köhler 1928). In the latter varieties the reaction may perhaps be interpreted as one of hypersensitiveness, but this conception cannot be applied when the host is uninjured and the parasite alone is killed.

With certain facultative parasites there are defensive mechanisms in resistant hosts which, while not preventing initial invasion, preclude the fungus from spreading extensively in the tissues. *Stereum purpureum*, the cause of silver-leaf disease of plum trees, commonly attacks the variety Victoria but only rarely the variety Pershore, infection occurring through exposures of the wood. If spores alight on newly exposed wood of a susceptible variety a mycelium is established which grows extensively in the vessels at most times of the year. Accompanying the development of the fungus considerable quantities of gum are formed from the carbohydrates in the wood. Simultaneously, substances produced by the fungus diffuse into the healthy wood, and, carried up in the transpiration stream, cause unfolding leaves to become silvery in appearance through the development of abnormal air-spaces, and old leaves to become prematurely brown Sterile extracts of the fungus produce the same symptoms when injected into a healthy susceptible tree (Brooks & Brenchley 1931) Normally, the progress of the fungus in the wood of the variety Victoria is not impeded, and sooner or later most of the wood of the trunk is invaded with the result that the tree dies. On the other hand, with the variety Pershore there is usually such profuse gum formation in the initiation of infection that the fungus appears to be unable to penetrate the concentrated layer of gum on the periphery of the invaded zone; the parasite is consequently occluded at an early stage (Brooks & Storey 1923, Brooks & Moore 1926) and subsequently dies. Such a protective mechanism is called a gum-barrier. During the summer, when metabolic activity of the host is greatest, the variety Victoria behaves like Pershore to initial invasion, i e. it is resistant to infection. Furthermore, the variety Victoria occasionally recovers from this disease, especially after a hot dry summer. If such a tree is examined a heavy accumulation of gum around the invaded tissues is found, which apparently the fungus cannot penetrate; again the parasite is occluded and dies in the course of time. It is suggested that the enhanced metabolism of the tree under such conditions enables it to prevent further progress by the fungus.

With *Armillaria mellea*, a root parasite of many kinds of trees, the defensive mechanism of a resistant species is more obscure (Thomas 1934; Leach 1939). In a susceptible species a branch of a rhizomorph penetrates the uninjured bark as a single unit and on reaching the cambium spreads rapidly as a compact strand in this region, at the same time invading the wood and inner bark in a more general way. In a resistant species penetration of the outer bark occurs similarly but extensive growth of the parasite along the cambium does not take place and there is only limited development in the wood. On the periphery of the invaded wood there is dense accumulation of gum and there is also a newly formed zone of cork on the margin of the affected bark. A strictly localized lesion is thus established beyond which the fungus does not usually spread; eventually the lesion may be

healed by growth of the tissues around the periphery. It is tempting to suppose that the accumulation of gum and the formation of cork are defensive mechanisms, although it has been suggested that they are only incidental to the secretion of a toxin by the host, which prevents further development of the fungus. Even in a susceptible host new zones of cork are formed in the inner bark, but these are readily penetrated by the advancing mycelium. It must also be pointed out that when the localized lesions on a root are very numerous the host resistance some-times breaks down, and the fungus then behaves as an active parasite.

The formation of cork-barriers or suberization of the membranes in non-woody tissues is often associated with the inhibition of extensive development of fungus parasites, as, for example, in varieties of apple trees resistant to canker caused by *Nectria galligena*. Sometimes, however, cork-barriers are an imperfect means of protection, for the fungus may evade them. Furthermore, their formation is greatly dependent upon the state of metabolism of the host, and under some condi-tions a normally resistant variety of apple tree, such as Bramley's Seedling, becomes susceptible. We do not know, however, how alterations in metabolism induce such changes in resistance. If the immediately exciting cause of the develop-ment of a cork cambium were ascertained progress towards elucidation would be made. For the formation of cork below wounds the liberation of hormonal sub-stances in the tissues is invoked.

Leaf spots caused by fungi are often delimited by a suberized zone of cells, leading sometimes to the falling away of the affected tissue and the well-known 'shot-hole' effect. Whatever interpretation is placed on the formation of such corky layers they certainly seem to prevent widespread ramification of the fungus. On the other hand, certain fungus leaf spots, although circumscribed in size, are not de-limited by cork-barriers, and there seems to be no obvious impediment to extensive spread of the parasite. In such leaf spots, apart from some obscure reaction by the host, perhaps further growth of the fungus is stopped by accumulation of staling products arising from its own metabolism.

Wilts of herbaceous plants are often induced by fungi which invade the roots from the soil and enter the vascular tracts. In varieties of flax susceptible to wilt caused by *Fusarium lini* the fungus invades the cortex and enters the vessels. In resistant varieties progress of the fungus is limited and appears sometimes to be stopped by suberization of the cell walls or the formation of a zone of cork in the cortex; the fungus is localized and no wilt is induced (Tisdale 1917). In susceptible plants the fungus grows extensively in the vessels in which small amounts of gummy substances are deposited, although it is doubtful whether the transpiration stream is appreciably impeded thereby. It is generally believed that wilting is caused by a toxin secreted by the fungus, especially as certain changes are induced in the leaves before wilting occurs. In a resistant variety the amount of toxin produced by the fungus is greatly reduced because of the localization of infection, and its formation may perhaps be inhibited or neutralized by some property of the host cells. In tomato wilt due to *F. lycopersici* there is strong evidence that a polypeptide

substance, lycomarasmin, of known composition, is the immediately inciting cause of wilting (Clauson-Kaas, Plattner & Gäumann 1944; Gäumann & Jaag 1946), which is brought about by destruction of the semi-permeability of the plasma membrane of the leaf cells. The fungus does not normally enter the vascular system of highly resistant varieties, which contain substances that depress the growth of the fungus *in vitro* (Gottlieb 1943). One might expect therefore that its development in the root would be impeded. There is a difference of opinion, however, as to whether resistance is localized in the root or whether it is a property of the plant as a whole. A resistant variety grafted on an infected susceptible one succumbs to wilt, whereas a susceptible variety grafted on a resistant one under conditions suitable for root infection remains unaffected (Heinze & Andrus 1945), these results imply that resistance is a property only of the roots. On the other hand, more recent investigations indicate that the fungus, even when artificially introduced into the vessels of a resistant variety, rarely induces wilting although growing extensively therein (Snyder, Baker & Hansen 1946), this implies that the shoot as a whole is resistant to wilting. Whether resistance is actually localized in the root or not one concludes as regards a resistant variety that the fungus is unable to produce sufficient toxin to cause wilting or that the toxin is either inactivated by the host or is without effect upon it. In the wilt of hops caused by *Verticillium albo-atrum* it has been shown that in resistant varieties the fungus develops extensively in the vessels without inducing wilt (Keyworth 1947).

Few examples are yet known in which resistance to fungus invasion is associated with particular substances of known composition, although there is increasing evidence that the resistance of certain plants to attack by *Phymatotrichum omnivorum* is associated with the presence of specific alkaloids in their underground organs (Greathouse & Rigler 1940). The most clearly established example of this kind is the resistance of red- and yellow-skinned onions to smudge and neck-rot caused by *Colletotrichum circinans* and *Botrytis allii* respectively, white varieties being susceptible to both diseases. Catechol and protocatechuic acid, associated with the pigments in the dead outer scales of the coloured varieties, are inhibitive in high dilution to the growth of these fungi (Link & Walker 1933). If, however, the dry pigmented scales of these resistant varieties are removed the bulbs become susceptible. It has been suggested that under moist conditions catechol and protocatechuic acid pass in solution to the exterior of the dead outer scales of the coloured varieties, thereby preventing the growth of these fungi. In this connexion it may be noted that *Aspergillus niger*, which rots both coloured and white onions, is not checked in growth by extracts from the pigmented scales.

Resistance to infection by fungus parasites is rarely absolute under all conditions; it is usually subject to modification, as has already been pointed out. It may be worth while, however, to discuss further the changes in resistance that can be brought about in a variety of ways, bearing in mind that in any parasitic attack the conditions of life of both host and parasite and also the relations between the two organisms have all to be considered.

Little attention has been paid hitherto in plant pathology to the influence of mass infections as distinct from sporadic ones. But where resistance is not pronounced a large number of infections may overcome the defensive mechanism of the host whereas a few have no adverse effect. This seems to be the explanation of the development of warts on certain varieties of potatoes, described as 'immune' in the field to *Synchytrium endobioticum*, when they are subjected to intensive infection in the laboratory (Glynne 1926). Under such conditions the concentrated inoculum apparently overcomes the normal resistance. Again, in the chocolate spot disease of beans caused by species of *Botrytis*, a heavy inoculum has an aggressive effect, whereas, under similar favourable environmental conditions, a sparse inoculum produces only slight infection (Wilson 1937).

Changes in environmental conditions such as temperature, light intensity, water supply, and the mineral nutrition of the host often affect the degree of resistance to specific parasites, but it is usually impossible to analyze the complex sufficiently to give guidance in interpretation. With the cereal rusts abnormal conditions of temperature and light may change the host's reaction from resistant to susceptible, or vice versa. Excessive nitrogenous manuring of the host often increases susceptibility to attack by rust fungi whereas additional potash sometimes reduces it. A striking effect on susceptibility to powdery mildew of wheat and to certain other parasites is shown by the addition of minute quantities of lithium salts to the soil in which the hosts are growing, such plants being rendered more resistant (Spinks 1913; Kent 1941). The lithium salts are known to be carried to the leaves but the manner in which they confer resistance is still obscure. Other changes in metabolism of the host may also have a striking effect on susceptibility or resistance. For instance, in the United States *Fusarium graminearum* causes a serious seedling disease of wheat at high temperatures and of maize at low temperatures. This difference has been correlated with the type of metabolism of the hosts at such temperatures (Dickson, Eckerson & Link 1923; Dickson & Holbert 1928). At low temperatures wheat seedlings resist attack because the cell walls are well provided with cellulose thereby preventing ready penetration by the hyphae, whereas at high temperatures the cell walls at this stage of development consist chiefly of pectic substances which are easily disintegrated by the fungus. The metabolism of maize seedlings in relation to temperature is just the opposite of that in wheat, hence susceptibility at low temperatures and resistance at high ones. In this example changes in metabolism profoundly alter the nature of the cell walls, but in many kinds of parasitic attack metabolic changes in the host have a much more obscure influence.

The genic difference between a resistant and a susceptible variety may be simple or complex, but its expression in the plant is frequently modified by environmental and metabolic conditions. Knowing that resistance and susceptibility to specific parasites are definite hereditary entities the plant breeder can plan his programme accordingly, although he has constantly to bear in mind the possibility that new physiologic races of the parasites may arise in the course of time.

SOME EFFECTS OF HOST-PLANT PHYSIOLOGY ON RESISTANCE TO VIRUSES

BY F. C. BAWDEN

Although the subject for discussion is the physiology of disease resistance, I propose to take a more restricted topic and to deal only with variations in the degree and type of resistance shown towards viruses by susceptible hosts. I do this because we know a little of some of the conditions that affect the reactions of susceptible plants, but we know nothing of the causes that render most plants immune from most viruses. With viruses, as with other kinds of pathogens, immunity is the common state of plants and susceptibility is the exceptional. Indeed, all uninjured plants appear to be immune from all viruses, and though there are a few viruses that can infect a wide range of different plants through suitable wounds, most viruses have only a narrow host range even as wound parasites. Presumably immunity is conferred on most species because their cells either contain substances that are antagonistic towards individual viruses or they lack substances that are essential for multiplication of the viruses. Extracts of many plants do contain substances that act as inhibitors of infectivity when mixed with viruses *in vitro*, but whether such substances play any part in conferring resistance *in vivo* is uncertain. We know nothing of the growth requirements of any viruses that attack flowering plants, but it is perhaps worth noting that there are bacterial viruses that lyse certain bacteria only when specific amino-acids are supplied to the culture media.

When we consider the types of resistance shown by susceptible hosts towards viruses, we find some that are common to other forms of infectious diseases but also additional ones peculiar to virus diseases. This could be expected, for viruses possess an unusual combination of properties. They are the only pathogens that are both obligate and wound parasites. For their spread from plant to plant most of them depend on the activities of certain vectors, of which the most common are leaf-sucking insects. A further peculiarity is their ability to cause systemic infections, for in most hosts viruses spread from a single entry point to invade all the vegetative parts of the plant that are still actively growing. It is this ability to permeate the whole system of infected plants that gives us with virus diseases the only authentic examples in plant pathology of acquired resistance to disease and provides perhaps the biggest contrast between virus and other types of disease. There are two rather different types of behaviour that come under this heading, both of which simulate conditions usually considered more typical of diseases of animals than of plants.

The first type is concerned with the recovery of plants from an initially acute disease, and is most vividly shown by diseases of the ringspot type. When tobacco plants are infected with tobacco ringspot virus, the inoculated leaves soon develop severe local lesions and within 10 days the young leaves are also acutely necrotic. At this stage, the reaction of the plants is so severe that it seems as though they could not survive, but within a short time they produce new leaves showing few or no symptoms and after some weeks the plants may look like uninfected ones. If

again inoculated with the ringspot virus, these plants do not develop any new symptoms, and if propagated vegetatively by cuttings, they continue to be resistant to the ringspot disease indefinitely. This sequence of recovery from an acute disease, followed by a subsequent resistance to it, is not brought about by antibody production in the host as are the superficially similar conditions in animals. The virus is present all the time in the leaves of recovered plants, and causes the typical disease if transferred from them to uninfected tobacco plants, but it is present in a lower concentration than during the acute stage of the disease. It seems that cells which are well developed before they become infected are in a metabolic state in which they allow virus multiplication to proceed extensively and in which they react severely to the presence of the virus. Infection of cells early in life apparently alters their constitution so that they cannot support such a large virus population and can tolerate its presence.

The second type of resistance plants can acquire against virus diseases simulates the process of vaccination of animals, whereby protection against a serious pathogen is conferred by earlier infection with a related but innocuous one. But here again, despite the seeming similarity, the process in plants differs fundamentally from that in animals. Antibody-production by the host is not involved, and the resistance in plants has its origin in the fact that individual strains of a virus are antagonistic one to another. Most plant viruses occur in strains of different virulence, which can be distinguished from one another by the type and severity of symptoms they cause in different plants. Potato virus X, the cause of mild mosaic, can be used as a convenient example, for the behaviour of the various strains of this virus in different hosts illustrates many of the different phenomena influencing resistance. The adjectives virulent and avirulent cannot strictly be applied to strains of this virus without specific reference to a host plant as well, for one strain may cause a serious disease in some hosts but not in others. Virulence is not an intrinsic property of a strain, but is only a reflexion of a host-parasite interaction. Whether one regards a pathogen as highly virulent or a host as very susceptible is largely a matter of convenience rather than any true distinction. Nevertheless, by their reaction in plants such as tobacco and *Datura stramonium*, the strains of virus X can be conveniently graded in order according to the severity of symptoms they cause. These symptoms range from acutely necrotic diseases of the ringspot type, in which affected plants are crippled, through bright mosaics to barely perceptible mottles, conditions that may have no obvious effects on the size and vigour of the plants. These different symptoms do not merely reflect the different virus contents achieved in the hosts by different strains, for some strains that cause severe diseases occur in smaller amounts than others that cause only slight symptoms. Acquired resistance is easily demonstrated by infecting *D. stramonium* seedlings with a strain of virus X that causes slight or no symptoms, and then 10 days later inoculating them a second time with one that causes ringspot symptoms; the second inoculation produces no further symptoms, whereas uninfected plants of the same age will become severely diseased. Only cells that are actually infected with one strain of the virus are resistant to infection with

a second, and in plants where infection is not yet fully systemic, a second strain will become established and produce its characteristic effects on uninfected parts but not on the others. The mechanism involved is far from understood, but it does not seem to be simply that there is only a limited quantity of one particular virus that a cell can produce, for strains that reach relatively low concentrations are effective in protecting plants against those that reach higher concentrations. It is perhaps more likely that there are only a limited number of sites of virus-multiplication in a cell and if these are occupied by one strain another cannot become established.

It is in potato plants that the word virulent becomes meaningless for strains of virus X, because in different varieties one and the same strain can cause diseases differing widely in severity. These various reactions include types of behaviour that simulate resistance. One of the commonest is hypersensitivity, which in one form closely resembles the hypersensitive reactions of cereals to those other obligate parasites, the rust fungi. The reactions of the initially invaded cells are so violent that they die rapidly and the virus is prevented from spreading from the site of infection. In such plants the only symptoms are necrotic local lesions, and most of the plant remains unaffected. Sometimes, however, there is a systemic form of hypersensitivity. In spite of local necrosis, the virus is not localized, but invades and kills the whole plant. This obviously is the ultimate point of susceptibility for an individual, but is, nevertheless, a property that confers resistance on a stock as a whole. Potato varieties that react in this manner to strains of virus X are rarely found infected in the field, whereas other varieties are almost universally so. The death of the individual rids the stock of the virus, whereas plants that are not killed remain as sources of infection for their neighbours and spread continues until the whole stock is affected. Hypersensitivity is inherited in a simple Mendelian manner, but of the physiological reactions that determine it nothing is known. Different genes confer hypersensitivity towards different strains of the virus, so that one variety may be hypersensitive to strain A but not to strain B, and another may reverse this.

Short of hypersensitivity, potato plants may react to different strains of virus X in many different ways, from conditions in which necrotic symptoms predominate, through severe mosaics, to slight conditions in which no clear leaf symptoms are produced and the growth of the plants is little affected. This variation in degree of tolerance to the presence of viruses is a feature of different varieties of most species of plants, and tolerance reaches its highest expression in carrier varieties which show no symptoms when infected, though they may contain more virus than intolerant varieties that show severe symptoms. A high degree of tolerance is a feature of considerable value to a variety and is a property that simulates resistance, though tolerant varieties may be, and often are, more susceptible to infection than intolerant ones. Tolerance to some viruses is affected by the conditions under which host plants are grown, and infected plants may look healthy in one environment but not in another. The potato variety Majestic, for example, when infected with certain strains of virus X, shows a definite mosaic at temperatures below 20° C but above this becomes symptomless.

The same potato variety may be hypersensitive to one strain of virus X, develop a non-fatal disease with a second and tolerate a third. These different reactions, as in tobacco, are not correlated with the extent to which the different virus strains multiply in the variety, so that symptoms do not result simply from a weakening of the plant because of the production of virus at the expense of normal cell constituents. Whether a variety becomes diseased or tolerates a virus strain, therefore, appears to depend on the occurrence of secondary reactions between some specific active groups possessed by the different virus strains and certain cell constituents. The only cell constituents known to affect the type of reaction are other virus particles. We have already seen that the presence of a related virus can prevent a second from producing its characteristic effects, but this antagonistic effect is restricted to strains of one virus. Unrelated viruses are often synergistic and the presence of two, which a plant can tolerate singly, can cause severe symptoms. For example, strains of tobacco mosaic virus and potato virus X, which singly cause only slight mottling in tomato plants, together produce streak, an acute necrotic disease.

So far we have largely considered resistance to viruses only as it is indicated by the degree and type of symptoms shown by infected plants. There are, however, other ways in which plants vary in resistance; susceptible hosts differ both in the ease with which they become infected and in the extent to which they permit virus multiplication once they have become infected. Dandelion yellow mosaic virus which attacks both dandelion and lettuce plants affords a good example of these differences. The dandelion is much more resistant than lettuce and inoculum that will readily produce an infection in lettuce fails with dandelion. The dandelion also seems to be a less favourable host for virus multiplication. No experimental transmissions of this virus from infected to healthy dandelion plants have been achieved; occasionally the virus has been transmitted from dandelion to healthy lettuce and from infected lettuce to healthy dandelion, but from infected to healthy lettuce it can be transmitted readily.

Similar, though less striking, differences in susceptibility to infection by leaf-roll virus and virus Y are found between different potato varieties. When varieties are exposed to equal chances of infection by the two viruses in the field, ten times as many infections may occur in some varieties as in others. And resistance to infection with one virus is not correlated with resistance to another; nor is it correlated with any varietal differences in susceptibility to aphis infestation, but can only be attributed to specific reactions between the viruses and the tissues of the different varieties. In conditions at Rothamsted, in which less than 10 % of Arran Banner plants contracted leaf-roll or virus Y, Arran Pilot became 70 % infected with virus Y and 10 % with leaf-roll, whereas Arran Consul became 50 % infected with leaf roll and 13 % with virus Y. Controlled transmission tests with virus Y using aphides under glass gave results duplicating the relative susceptibility as found by field trials; these tests also showed that resistance to infection is not necessarily correlated with hosts that are incapable of maintaining a large virus population

once they have become infected. The virus content reached in Arran Banner plants, one of the varieties most resistant to infection, is higher than that in other varieties which become infected more easily.

These and other phenomena suggest that different amounts of virus may be needed to cause infection in different hosts. The concept of varying minimal dosages, however, conflicts with other evidence. The form of dilution curves, for example, indicates that infection is caused by single virus particles, and all our evidence suggests that each infection is a local and independent event and that one does not occur from the accumulation of separate sub-minimal doses. To reconcile these apparent contradictions, it seems necessary to assume that cells of different varieties contain varying amounts of substances that can combine with and neutralize entering virus particles, or in some way prevent them from becoming established and multiplying. If this is so, then, although one particle may be enough to infect any susceptible cell, the chances of one particle actually doing so will vary with different cells. Only one particle will be directly responsible for any one infection, but in different cells different numbers will need to be introduced to ensure that one becomes established in whatever conditions are required for multiplication.

It is probable also that different tissues of one host differ in their susceptibility to infection. With a few viruses, such as curly-top of sugar beet and maize streak, infection seems to occur only when the virus particles are placed directly into the phloem, although with maize streak at least the virus occurs later in other tissues. With mechanically transmissible viruses there is less need to place the infecting dose in a particular tissue, but with these also the method of inoculation is important. Whether or not a plant becomes infected by a given concentration of virus depends sometimes on how the inoculum is introduced. Potato plants, for example, do not become infected with virus *A* when rubbed with sap from infected plants unless some suitable abrasive, such as 400-mesh carborundum or kieselguhr, is used. Other viruses which can be transmitted without this aid also cause many more infections when applied with an abrasive, the effect being to increase the numbers of local lesions obtained by approximately the same amount as though the virus content of the inoculum were increased a hundred times with altering the method of inoculation. Clearly abrasives cannot be increasing the virus concentration and must be acting by increasing host susceptibility. They may act in part merely by producing more wounds and so supplying the viruses with more entry points, but it seems likely that they expose cells not made available to the viruses by ordinary rubbing. The injury caused to *Nicotiana glutinosa* leaves by rubbing without abrasives is largely confined to trichomes, whereas with an abrasive the permeability of the deeper epidermal cells is affected, and these cells appear to be more susceptible than the trichomes, in the sense that a single virus particle when introduced has more chance of becoming established and multiplying.

In addition to causing local lesions, rubbing with abrasives sometimes affects the early course of systemic infections, leading to earlier and more severe symptoms

than applying the same inoculum without an abrasive. Majestic plants infected with potato virus Y, for example, show systemic symptoms up to a week earlier when an abrasive is used than when it is not and both the necrotic and mosaic symptoms are more intense. These differences probably merely reflect the different initial amounts of virus established in the inoculated leaves by the two methods. With a high initial concentration, virus would be able to move from the inoculated leaf sooner than with a lower concentration, and so reach uninoculated tissues when these are younger and more susceptible to its effects. For a phenomenon similar to that known with the rust fungi as mature plant resistance is common in virus diseases, many plants becoming increasingly resistant to infection and to the effects of infection with increasing maturation. Increasing resistance with age is very clearly shown by tomato and *Nicotiana glutinosa* towards tomato bushy stunt virus. Young tomato seedlings are extremely susceptible, and within a few days of inoculation develop many local lesions, both chlorotic and necrotic. Systemic infections develop within a week of inoculation, are acute and may soon lead to the death of the whole seedling. In rather older plants, fewer local lesions are obtained and systemic symptoms are less uniformly distributed over the whole plant, consisting of isolated chlorotic or necrotic spots, and in still older plants infection is restricted to occasional local lesions. In *N. glutinosa* there is a gradient of increasing susceptibility from oldest to youngest leaves and the type of lesion, as well as the number, varies on leaves of different ages. On the young leaves the lesions are large and wholly necrotic, consisting of a brown centre surrounded by a black ring. On the old leaves the lesions are small and consist of a white necrotic pin-point, which may have a chlorotic halo. On the intermediate leaves, the lesions are intermediate in size and type. The difference in susceptibility does not arise because leaves of different ages injure to different extents when rubbed and so produce different numbers of wounds, for when tobacco mosaic virus is inoculated a gradient in the opposite direction is obtained, more lesions being produced on the older than on the younger leaves. One imagines that an injury that would permit particles of one virus to enter would also permit those of another, and the effect seems to be produced by age altering the metabolism of the cells so as to prevent the successful establishment of tomato bushy stunt virus in injured cells but not tobacco mosaic virus.

In addition to age, the conditions in which plants are grown also greatly affect susceptibility. Tomato and *N. glutinosa* plants are much more susceptible to tomato bushy stunt virus, and tobacco plants to tobacco necrosis viruses, during the winter than during the summer. Infection not only occurs more readily but the virus content of infected plants is also higher. This seasonal effect is largely explained by the fact that light intensity greatly influences susceptibility, and by raising plants during the summer under shade their susceptibility to infection with these two viruses, as measured by numbers of local lesions, can be increased by more than ten times. Part of the increased susceptibility produced by shading may be simply a mechanical effect. Leaves of plants raised in shade are more fragile than those

exposed to bright light, and no doubt suffer more injuries during the course of inoculation. However, this effect can hardly explain the greater virus content of systemically infected leaves, and it seems that some additional factor is also involved. Plants that have been raised under normal glasshouse conditions can have their susceptibility to infection with several viruses greatly increased merely by placing them in the dark long enough for the carbohydrates to disappear. The susceptibility of bean plants to a tobacco necrosis virus is increased five times by placing them in darkness for the 24 hr. before inoculation, a treatment that produces no obvious effect on the fragility of the leaf or susceptibility to injuries during inoculation. Similar results in increasing susceptibility to aucuba mosaic virus are produced by placing tobacco plants in the dark, though more than 24 hr. are needed to produce the maximum effect. Periods of darkness affect susceptibility to infection only when they are applied to plants before they are inoculated; post-inoculation treatment has no effect or decreases the numbers of lesions obtained. It appears that the successful establishment of infection occurs in two stages, the first consisting of the entry of virus particles into cells and their establishment at sites of multiplication, and the second being the processes that lead to multiplication and the production of symptoms in intolerant hosts. The first stage is adversely affected by the presence of large quantities of products of photosynthesis. It is unknown whether these products confer resistance by reacting specifically with virus particles to render them non-infective or act mechanically by increasing cell turgor or interfering with the attachment of particles at multiplication sites, but when mixed with viruses *in vitro* sap from plants in light and dark has the same inhibitory power.

It is clear from these results of varying the conditions of illumination that the physiological condition of plants greatly influences their resistance to viruses, affecting both the ease with which infection occurs and the extent to which the viruses permeate the plants and multiply. In addition to the rate of photosynthesis, nutrition of the host also affects resistance. The interpretations of these effects, however, are rather complicated because of the effects of nutrition on the size of the plant. If one assesses susceptibility by the number of lesions per leaf one may conclude that nutrition has different effects than if the number per unit area is considered. Also, when using virus multiplication as an index of susceptibility, it makes great differences whether one considers concentration achieved in a given volume of plant extract or the total virus population of an infected plant.

In experiments we have made to test the effect of nutrition of the host on susceptibility of tobacco to strains of tobacco mosaic virus, we have found that phosphorus is more important than nitrogen or potash in increasing susceptibility to infection and in increasing virus multiplication, but it is also the most effective in increasing plant size. Assessing susceptibility by numbers of lesions per leaf, the addition of phosphorus increased susceptibility by four to five times, whereas assessing it by lesions per unit area the addition increased susceptibility by less than 50 %. Similarly, in considering effects of nutrition on virus multiplication, assessing susceptiblity by virus per ml. of sap, phosphorus approximately doubled

the virus content, but assessing it by virus per plant it gave an increase of ten times.

The most reasonable index of susceptibility for virus diseases is probably the individual plant rather than any standard area of leaf or volume of extract, for in natural conditions most viruses cause systemic diseases and only one initial infection is needed to infect the whole plant. This is a difference between systemic and non-systemic diseases that makes size of the plant itself an important factor in determining susceptibility. Other things being equal, a large plant will stand more chance of becoming infected than a small one, because its greater surface area renders it far more likely to collect infective insects or come into contact with other sources of infection. When infection has occurred, the virus has more plant tissues to invade in the larger plant and so a greater virus population will result from a single infection, and chances for further spread will be correspondingly increased. Thus small size provides plants with a convenient disease-escape mechanism that in the field may simulate resistance. With virus diseases that are insect-transmitted, susceptible plants may also escape infection and appear to be resistant if they are unattractive to the insect vectors or if they grow in conditions that reduce the chances of visits by vectors. The last feature has for long been used to reduce losses from potato virus diseases, and the growing of seed stocks in conditions unfavourable for the peach aphis will continue to be the basis for control until some other property conferring resistance is shown to be equally effective and cheaper.

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The haemoglobin of *Daphnia*

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Daphnia is often coloured pink or red by haemoglobin in solution in the blood. This applies to pond-living but not to lacustrine species. A *Daphnia* species may vary from red to colourless in different ponds or in the same pond at different times   In the laboratory individual *Daphnia* can be seen to lose or gain haemoglobin in the course of a few days

Daphnia loses haemoglobin in well-aerated water and gains haemoglobin in water containing little dissolved oxygen

Abundance of parthenogenetic young may be taken as a criterion of good nutrition, judged by this standard, good nutrition alone does not result in abundance of haemoglobin Nor is chlorophyll in food a cause of haemoglobin production

There is a haemochromogen in solution in the intestinal fluid of *Daphnia* Like helicorubin in the snail it shows reversible oxidation. In quantity it is proportional to the haemoglobin of the blood, which suggests that it may be an excretory product of haemoglobin Yet it occurs in a lake plankton species lacking the blood pigment

Occasionally one of the two excretory shell glands of *Daphnia* contains concentrated haemoglobin This pathological condition may be an indication that haemoglobin is normally excreted as such by the shell glands.

The presence of haemoglobin in the blood of *Daphnia* suggests a respiratory function. The increase in quantity of the haemoglobin in response to oxygen deficiency, just as in man, supports this thesis. Nevertheless, there appears to be no such function Animals whose haemoglobin is functionally inactivated with carbon monoxide are as vigorous and survive as well as untreated animals, at all concentrations of air dissolved in the water

Haemoglobin is present in the parthenogenetic eggs of *Daphnia* as well as in the blood Respiratory conditions in the brood pouch of parthenogenetic females are not good. This suggests an importance of haemoglobin in parthenogenetic development Experiments in which the haemoglobin was functionally inactivated by carbon monoxide showed that the respiratory pigment of the egg does have a favourable influence on late stages of the parthenogenetic embryo.

Fertilized eggs, in ephippia, contain no haemoglobin. Nevertheless, they develop as well in water deficient in oxygen as in aerated water.

1. Introduction

Those species of *Daphnia* which live in ponds or ditches are often pink or even red. Swammerdam (1758) described the colour of the 'Arborescent Water Flea' as being 'like that of beef, which has been some time steeped in water'. He relates how he

saw so many in one pool 'that the water appeared as if changed into blood; which, indeed, terrified me at first'. The aspect of such ponds is well described by Baird (1850). The colour is due to haemoglobin in solution in the blood of the animals. Lake-dwelling species of the genus have no pink tint.

In the Crustacea haemoglobin is widespread among Entomostraca, but it is unknown in Malacostraca. Ray Lankester (1871) discovered the haemoglobin of *Daphnia* and *Chirocephalus* with the spectroscope. The blood of Apus (*Triops*) is red with haemoglobin (Regnard & Blanchard 1883). Lochhead & Lochhead (1941) found the pigment in *Artemia* and I have found it in the ostracod *Cypria ophthalmica* (Jurine). The blood of the conchostracan *Limnadia* is described as bright red (Klunzinger 1864) and haemoglobin may well be responsible. Haemoglobin occurs in the blood of some parasitic copepods (van Beneden 1880; Fox 1945b) but has not been found in free-living members of this order. It is found also in a parasitic cirripede (Pérez & Bloch-Raphäel 1946).

The fact is familiar to aquarium keepers who use *Daphnia* as fish food that water fleas are sometimes red, sometimes pink, and at other times have hardly a trace of reddish tint, or none at all. But it is not known to most zoologists that any one of the several pond species may be either red or colourless, and still less is it known that one and the same individual can gain or lose haemoglobin to a surprising extent in the course of a few days. There is, indeed, no other animal known which shows these great changes. Nor does *Daphnia* appear to suffer in vigour from being anaemic. Clearly it is of importance to discover the factors responsible for this rapid and considerable synthesis and loss of haemoglobin. It is equally interesting to know the function of a haemoglobin so variable in quantity, and to find out where it is formed and how it is got rid of when it diminishes.

2. SITUATION, PROPERTIES AND FLUCTUATION OF HAEMOGLOBIN

(1) *Haemoglobin in blood and eggs*

In Britain there are three common species of *Daphnia*, namely *D. magna* Straus, *D. pulex* (De Geer) and *D. obtusa* Kurz, which are found in ponds or ditches (Scourfield & Harding 1941; Scourfield 1942). The haemoglobin content of these species varies widely. There are other species, inhabiting open waters or lakes, which are not visibly coloured by haemoglobin. In the species named above, the haemoglobin is localized in two tissues: it is found in solution in the blood plasma and it is present in the eggs. There is no haemoglobin in the muscles. A pink *Daphnia* examined under the microscope with a spectroscopic ocular shows the α- and β-bands of oxyhaemoglobin. The animal can be bled white by teasing it with fine needles, if then all blood is washed from the interstices of the muscles, no trace of the absorption bands remains. When now pyridine and sodium hydrosulphite are added, no haemochromogen band appears, proving that not only is there no haemoglobin in the muscles but the latter contain no other haem compounds detectable in this way. This is not unexpected since the limb muscles of other

crustaceans, such as crabs, contain so little haem that it can only be shown clearly with pyridine in considerably greater thickness of muscle than is available from one bled *Daphnia*.

Teissier (1932) discovered that the eggs of *D. pulex* in the brood pouch contain haemoglobin. As he points out, this is the only known occurrence of haemoglobin in an ovum. Since then an analogous case has been reported. the eggs of the marine echiuroid worm *Urechis caupo* contain a pigment, urechrome, which a vacuum reversibly changes from yellow to pink (Horowitz 1940). In *Daphnia pulex*, *D. obtusa* and *D. magna* the parthenogenetic eggs are more often than not coloured green by a carotenoid-protein pigment. The tint varies very considerably, no doubt with the particular algal food of the mother, and Teissier has shown that feeding *Daphnia* artificially with carotene-free 'farine lactée' produces eggs without the pigment. In these eggs the pink colour of oxyhaemoglobin is visible, and *Daphnia* in certain ponds has such pink eggs. But in all *Daphnia* of the three species named, no matter how green are the eggs, the bands of oxyhaemoglobin can be seen in a single egg with the microspectroscope. This is so even when the blood is so pale that no bands are visible in it when a single animal is examined.

(2) *Properties of the blood haemoglobin*

The following physico-chemical data are available on the haemoglobin of *Daphnia* blood.

(i) Its molecules appear to be of two sizes, with weights respectively one-half and six times that of mammalian haemoglobin (Svedberg & Eriksson-Quensel 1934).

(ii) The uptake of oxygen by *Daphnia* haemoglobin occurs at oxygen pressures which are not quite so low as in the case of other invertebrates. Yet, at the temperatures at which these animals live, the requisite pressures are much lower than for human haemoglobin at blood heat For example, the oxygen pressures (in mm. of mercury) for 50 % oxyhaemoglobin at 17° C are 3·1 for *D. magna* and 0·6 for *Chironomus riparius* in the absence of carbon dioxide; in the presence of 1 % carbon dioxide the value for *Daphnia magna* is 4·9. For man at 37° C and pH 7·4 the value is 27 mm. But the oxygen pressure in the water outside *Daphnia* need fall no lower than 28 mm. (1·18 ml. oxygen per litre at 17° C) to cause deoxygenation of the haemoglobin in the animal's blood, since there is a steep oxygen gradient across the body wall (Fox 1945*a*)

(iii) Species of the genus *Daphnia* have different haemoglobins, the wave-length of the α-band axis for the oxyhaemoglobins of *D magna*, *D. pulex* and *D. obtusa* being respectively 5766, 5764 and 5761 A (Fox 1945*b*, 1946).

(3) *Measurement of haemoglobin in the blood*

In order to estimate changes in the haemoglobin content of *Daphnia* blood, in nature and under laboratory conditions, a quantitative method was devised. This enables the haemoglobin concentration to be measured in single individuals. The

comparison standard consists of 0·2 ml. of blood from the worker's finger diluted in 75 ml. distilled water, with the addition of a trace of saponin for complete haemolysis and a drop of sodium bicarbonate solution to avoid breakdown to haematin. Of this solution, 50 ml. are put into a wedge-shaped optical glass trough, 10 cm. long, 4 cm. across at the wide end and 4 cm. high. A trace of octyl alcohol is added to the diluted blood to lessen the creep up the narrow end of the trough. The trough is placed before the mirror of a microscope. The optical disposition is as follows: daylight lamp with water-bath and ground-glass screen in front of it, trough, concave mirror, condenser screwed up, 16 mm. objective and × 6 eyepiece. The image of the haemoglobin solution is made to fill the upper third of the evenly illuminated field of vision in the microscope. Ten large *Daphnia*, taken at random from a population, are laid in a row, out of water but damp, on a microscope slide without a cover-slip. The tint of oxyhaemoglobin close to the base of the second antenna* of each animal in turn is matched with the standard diluted blood by sliding the trough to right or left. The trough stands on a paper scale so that its narrow end when seen through the microscope corresponds to 0, its wide end to 160 arbitrary units. (For pale populations the standard haemoglobin solution is diluted to one-half strength, the scale then reads from 0 to 80 units.) The values for each of the ten individuals are averaged and thus the *haemoglobin index* for the population is obtained.

Tests of the accuracy of the method were made by finding the haemoglobin indices of nine different populations, each in duplicate, that is, two lots of ten individuals were measured from each population. The results were 104 and 97, 93 and 86, 73 and 61, 60 and 56, 48 and 47, 46 and 44, 41 and 41, 40 and 39, 29 and 27. It follows that the error of the method is less than 10%. The method depends upon the constancy of the haemoglobin content of the observer's blood, but the small daily or longer-period fluctuations in haemoglobin content are not likely to be great compared with other unavoidable experimental errors. Obviously a mixed solution of dyes imitating the colour of haemoglobin would be preferable if such could be found. When the haemoglobin index had been determined the average individual size was estimated by measuring the length of each of the ten animals, from forehead to base of posterior shell spine, using an eyepiece micrometer with a 50 mm. objective. The number of eggs or young in each brood pouch was then counted, and various details of egg colour, food, fat, etc., were noted.

(4) *Variation of blood haemoglobin in nature*

The highest haemoglobin indices I have found in nature are 128 for *D. pulex*, 103 for *D. obtusa* and 118 for *D. magna*. These are, of course, averages of ten individuals, the highest single individual value was 150 for *D. pulex*. The size of

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* This position was chosen because a considerable thickness of blood space can here be seen by transparency without interference of the alimentary canal. This blood space is the front end of the 'loge intestinale' of Hérouard (1905), who by mistake gives it the name 'ventrale' on p. 221, line 11 from below.

D. pulex and *D. obtusa* is usually about the same but *D. magna* is generally bigger, the average length of individuals in the three populations whose haemoglobin indices are given above were 2·0, 2·2 and 3·3 mm , but *D. magna* may have an average length up to 4·5 mm. This means that while the indices of *D. pulex* and *D. obtusa* are comparable, in *D magna* the depth of blood measured is greater, so that this species has relatively less haemoglobin than its index would suggest. The lowest indices found in nature for all three species were less than 10, below which figure there is no measurable pink colour, so that no precise value can be given. If we suppose the index of such a population of *D. pulex* to be 10, it follows that the haemoglobin content of the blood in this species can vary 12-fold

The haemoglobin content of a population in a pond changes as time passes In one pond the indices for *D. magna*, at intervals from September of one year to April of the next, were 45, 57, 81, 56 and 42. In another pond *D. pulex* between August and December had successive indices of 36, 50, 62 and 72. In both cases the maximum change in half a year was twofold When there are two species in a pond their indices at one and the same time may differ considerably, although the sizes of individual are the same. In one case the index for *D pulex* was 40, for *D obtusa* 63 In other ponds, on the contrary, the former species had more haemoglobin than the latter. In yet another pond *D magna* had 81, *D. obtusa* 103, the latter species is much the smaller, so obviously its blood had a considerably greater haemoglobin content. But this is not a specific character, for in a different pond *D. obtusa* had much less haemoglobin than *D. magna*, even after allowing for their size discrepancy. Whatever it is in water or food that influences the quantity of haemoglobin, the factor acts differentially on two species in one pond. and its relative effect on two species differs in different localities Yet in a given pond two species can show parallel changes, for instance, in one case at bimonthly intervals the indices for *D. magna* and *D. obtusa* were 81 and 103, 56 and 97, 42 and 66.

3. CAUSES OF GAIN AND LOSS OF HAEMOGLOBIN

(1) *Previous work*

Fritzsche (1917) was under the impression that the red colour of *Daphnia* is due to carotene and that the redness is to be ascribed to good nutrition. Schultz (1928) accepted the opinion of Fritzsche and showed experimentally to his satisfaction that the red colour appears only in light and is never formed in cultures kept in darkness. Banta (1939) believed the red colour 'to be of the nature of intra-vitam staining obtained from the water'.

Verne (1923), recognizing that the red colour is due to haemoglobin, studied the cause of its synthesis. Many workers who have kept *Daphnia* alive in the laboratory have noticed that, in the course of a week or so, red populations get noticeably paler. Eventually the same individuals and their offspring become quite colourless. Verne started with *D. pulex* which had become 'incolores' in 8 days, I myself have never seen such a quick complete decoloration. He made three series of

cultures: (1) with 'débris de feuilles, ou des algues riches en chlorophylle, en macéra-tion dans l'eau. J'ajoutais des traces d'un sel organique de fer'; (2) 'Cultures de zooglées sans chlorophylle, ne montrant pas de fer décélable'; and (3) the last-mentioned medium with the addition of iron. At the end of 3 weeks (1) had haemoglobin, (2) and (3) had none From this he concluded that the appearance of haemoglobin depends on the presence of chlorophyll or of its breakdown products.

Verne gives no experimental details or quantitative data. I have quoted in his own words his statements of how the experiments were made because his con-clusion is far-reaching This is so both from the standpoint of biochemistry, implying that chlorophyll with its pyrrol groups supplies the building stones of protoporphyrin, and from that of medicine, since there have been proprietary preparations on the market claiming to benefit anaemia through chlorophyll derivatives. Indeed Verne, in a further publication (1924), elaborates a thesis derived from his *Daphnia* experiments that animals in general which are able to break down chlorophyll in their intestine as far as porphyrin use the latter for building up haemoglobin. He had not, incidentally, shown that *Daphnia* breaks down chlorophyll to a porphyrin.

(2) *Haemoglobin synthesis due to oxygen lack*

The first experiments I made were designed to test the effects of light and dark-ness on the haemoglobin content of *Daphnia* blood. In a typical experiment *D pulex* with an initial haemoglobin index of 61 was used. As usually happens in the laboratory, this value fell in the course of days, but it fell much more quickly in light than in darkness. After 21, 29, 34 and 45 days in light and darkness respectively the indices were 23 and 52, 16 and 53, 25 and 44, 20 and 36. But darkness or dim light did not always slow down the loss of haemoglobin; it sometimes resulted in a gain In one experiment *D pulex* started at 67; after 4 days in bright light and in shade the indices were respectively 66 and 87 In another case, starting with an index of 33, 4 days in bright light caused no change, whereas 4 days in shade raised the value to 59 This contradicts Schultz (1928) referred to above.

The effect of light and darkness on the haemoglobin content of *Daphnia* blood might be (a) a direct one on the animal, or (b) the result of more green algal food in the light, or (c) a higher oxygen content of the lighted cultures caused by algal photosynthesis and an oxygen deficit in the dark brought about by bacteria and by the *Daphnia* themselves.

Experiments showed that the dissolved oxygen content of the water does indeed affect the haemoglobin content of *Daphnia*. In one case the initial index of *D. pulex* was 62. The animals were divided into two lots, through one of which air was bubbled. The air was bubbled into a wide glass tube closed below with gauze and suspended in the vessel containing the *Daphnia*. The object of this procedure was to prevent the bubbles from hitting the animals. Next day the air saturation* of

* Dissolved oxygen was measured by the syringe-pipette micro-Winkler method of Fox & Wingfield (1938).

the water in this lot was 91 % and the haemoglobin index had fallen to 48. The other moiety was in a conical flask with little surface Respiration had reduced its air saturation next day to 9 %, while the index remained virtually steady at 65. The fall in haemoglobin due to aeration was in this case unusually fast.

Other experiments showed that oxygen lack not only may prevent the loss of haemoglobin but it can be a factor in its synthesis. In one experiment *D. pulex* had an initial haemoglobin index of 21. The population was divided into two halves, through one of which air was bubbled. After 4 days this had an air saturation of 82 % while the other half had a value of 21 %. The haemoglobin indices had become respectively 19 and 56. The amount of haemoglobin was thus more than doubled in 4 days by decreasing the available oxygen. Further experiments (e.g. (*b*) on p. 203) confirmed this result.

Since dissolved oxygen influences the haemoglobin content of *Daphnia* it seems probable that the oxygen content of the water is the explanation of the effects of light and darkness, the cultures in the light contained more oxygen, thanks to algal photosynthesis. I have been unable to find a direct effect of light on the amount of haemoglobin in *Daphnia* blood. For instance, the loss of haemoglobin by red *Daphnia* kept in pond water lacking algae is the same in light and darkness. Nor does green food affect the blood pigment, pale *D. obtusa*, some of which were fed on yeast and others on the green alga *Gonium pectorale*, both lots being kept in darkness for 5 days in water of a low oxygen content, gained haemoglobin to the same extent.

(3) *Influence of nutrition*

My results contradict the conclusion of Verne, for the animals which lost haemoglobin in the window were feeding on algae, while those which gained it in the dark had fewer or eventually no algae in their food. It was once doubted whether *Daphnia* can digest cellulose and thus feed on unicellular algae other than flagellates (Naumann 1921), but various workers have shown that *Daphnia* can be cultured on pure strains of algae (cf. Mortimer 1936, Lefèvre 1942). Algal cells can become colourless by digestion in the gut of *Daphnia* while the cell walls still retain their shape and their cellulose reaction (von Dehn 1930), the cell contents are leached out. The food of *Daphnia*, which in nature often consists of much dark detritus and a few algal cells, is seen under the microscope to be confined in the gut within a peritrophic membrane (Chatton 1920). The wide space in the front half of the midgut between this membrane and the gut wall is often coloured green with chlorophyll, or a breakdown product of chlorophyll, showing with a microspectroscope the characteristic intense absorption band at the red end of the spectrum. This pigment may be of a brilliant green and there is so much of it that it must accumulate gradually from the sparse algal cells scattered among the detritus in the gut. The green pigment is perhaps adsorbed on a colloid, which may be the swallowed secretion of the big labral glands, which Cannon (1922) showed not to be mucus. Be this as it may, *Daphnia* progressively losing haemoglobin in the laboratory very often has its gut lumen green with chlorophyll. In nature the pond

water may be a green soup of algae while the *Daphnia* in it has colourless blood. Yet this is by no means always the case; *Daphnia* in another green pond may be pink or red. And *Daphnia* in clear water containing few algae may have pale blood with little haemoglobin but bright green intestinal fluid. All this indicates that chlorophyll as food is not responsible for haemoglobin.

It has often been thought that red blood in *Daphnia* implies good nutrition. Fritzsche (1917) was of this opinion. It cannot, however, really be the case that red blood is just due to good food. In the light and dark experiments there was at least as much algal food in the light as bacterial in the dark. Yet it can be objected that the bacterial food may have been more nutritious, especially as the digestion of algal cell walls is apparently not easy. But there is an indication to the contrary, the lighted cultures were better fed, since they produced more eggs. In the first experiment quoted above (p. 200), the mean number of parthenogenetic eggs or young per mother after 21, 29, 34 and 45 days in light and dark respectively were 6·2 and 0·8, 4·6 and 0·7, 5·2 and 0·9, 3·4 and 0·6. There were more eggs in the light, and this was typical of other cases. It is well known that good feeding gives large broods of Cladocera. To take one instance, *D. pulex* with a mean egg count of 4·1 was kept in a laboratory window with and without abundance of an alga, *Chromulina Rosanoffii*, as food. After 8 days both lots were swimming actively but the mean egg numbers were 12·6 and 0. Thus, as judged by egg production, the lighted cultures in my first experiments were better nourished, yet it was those in the dark which retained or formed more haemoglobin.

Yet, much haemoglobin is not necessarily correlated with an impoverished state, for in nature red *Daphnia* may or may not carry numerous eggs. To take an example, a population of red *D. magna* in nature with a haemoglobin index of 90 had a mean egg number of 35. In another pond *D. magna* of index 86, which is sensibly the same, carried only an average of three eggs. Both observations were made in autumn. I have known individual *D. magna* with very pale blood bearing a record number of over 90 eggs in the brood pouch. Thus neither in nature nor in the laboratory is there any direct relation between number of young and haemoglobin content of the mother's blood.

Feeding does intervene in haemoglobin synthesis, however, to this extent that minimal nutrition is necessary, starved *Daphnia* does not produce the blood pigment under circumstances when well-fed animals do so. This was found in experiments in which pale *D. obtusa* was kept for 5 days (in the dark) in water of low oxygen content (a) with *Gonium pectorale* given daily as food, and (b) without this food. With the food haemoglobin was synthesized; without food there was a heavy mortality without any haemoglobin synthesis in the survivors.

(4) *Pond water and haemoglobin synthesis*

Pink or red *Daphnia* kept in the laboratory in water nearly saturated with air usually lose their haemoglobin, becoming gradually paler. In water deficient in dissolved air similar *Daphnia* lose haemoglobin much less rapidly, or they may

retain it. In other experiments, however, animals in aerated water did not lose haemoglobin, and those kept in partially anaerobic conditions increased their blood pigment, becoming redder. These experiments, resulting in the retention of haemoglobin in aerated water and its increase with slight oxygen deficiency, were made in water from a pond in which *D. pulex* was unusually red, the haemoglobin index was 128. This suggested that some other factor in the water in addition to oxygen deficiency may intervene in haemoglobin synthesis The idea was supported by experiments made with this same pond water, in the course of which the haemoglobin increased even when the amount of dissolved oxygen was not very low. For example, when the initial haemoglobin index of *D. pulex* was 37, after 5 days in water from this pond, as much as 84% saturated with air, the index rose to 47, while 5 days in the same water at 48% air saturation, which is not a very low value, raised the index to 66. There is apparently a factor in this water other than oxygen deficiency which stimulates haemoglobin synthesis.

Abundant rain-water falling into a pond with red *Daphnia* has on more than one occasion given the impression of causing the animals to become paler In one case I measured such an effect A water butt in a garden had a population of red *D. pulex* with an index of 91. The butt was half empty and I added enough tap water to increase the water volume by one-third Eight days later the haemoglobin index had dropped to 77. Apparently a haemoglobin-stimulating substance in the water had been diluted but perhaps the oxygen content of the water had merely been raised.

(5) *Haemoglobin synthesis promoted by duck faeces*

The species of *Daphnia* which can become red with haemoglobin are found in waters that are to a greater or less extent polluted with organic matter. Ducks are one of the sources of pollution. Accordingly experiments were made to discover whether duck faeces contain a substance that is able to stimulate haemoglobin synthesis.

Very pale *D. magna* with a haemoglobin index of 11, which had been feeding on algae for months in the laboratory window, were put into water with a suspension of duck faeces. Six days later their haemoglobin index was 54, while that of the stock culture in the window was unchanged The faeces suspension, owing no doubt to bacterial action, was only 39% saturated with air at the end of the 6 days, while the stock *Daphnia* culture was 140% supersaturated as a result of algal photosynthesis. Was it the oxygen deficiency or the duck faeces that had stimulated haemoglobin synthesis?

This question was studied as follows. Pale *D. magna* with an index of 17 were put into (*a*) a suspension of duck faeces, (*b*) water through which hydrogen was slowly and continuously bubbled (with the precaution mentioned above to avoid disturbing the animals), and to which algae were added for food. After 5 days the haemoglobin indices had risen to (*a*) 56, (*b*) 36, while the average air saturations, from six estimations made during the 5-day period, were (*a*) 26%, (*b*) 18%. The

haemoglobin had increased over threefold in presence of the faeces, but it had only doubled in the hydrogenated water, although the oxygen content of the latter was lower than that of the former. In another experiment the initial index was 36, after 10 days the indices became (a) 69, (b) 54 and the average air saturations, from two estimations daily, were (a) 22 %, (b) 16 %.

It is clear that duck faeces contain something which promotes haemoglobin formation over and above the effect of oxygen deficiency. Is this just better nutrition, or is it a specific substance?

4. BREAKDOWN AND EXCRETION OF HAEMOGLOBIN

(1) *Synthesis and breakdown*

In vertebrate animals the sites of haemotopoiesis are known, but not as yet in *Daphnia*. The precursor of haemoglobin might be a porphyrin, but I have been unable to detect porphyrin either in individual *Daphnia* or in mass extracts. In man, as in *Daphnia*, oxygen deficiency in the environment stimulates haemoglobin formation (Campbell 1926), and the same is true of fishes (Schlicher 1926; Ozolius 1936), but the causal factor acting in the bone marrow of man or the spleen of fishes is unknown, in *Daphnia* we could obviously not yet expect to know the train of causes between oxygen lack in the water and haemoglobin production in the animal.

In the vertebrates the haem of haemoglobin is continuously broken down to bile pigment and iron, and the bile pigment is excreted. Does *Daphnia* also get rid of its haemoglobin thus? I have not been able to detect any bile pigment in *Daphnia*. There are, however, two indications of other possible modes of exit of haemoglobin from the cladoceran body, namely, an occasional pathological condition of haemoglobinuria and the normal presence of a haemochromogen in the lumen of the gut.

(2) *Haemoglobinuria*

The excretory organs of *Daphnia* are a pair of relatively large, clearly visible maxillary glands, often called shell glands. As in all Crustacea these excretory organs are closed internally and they are bathed in blood. Klotzsche (1913) noticed that occasionally one of the two shell glands of *Daphnia* is bright red. A microspectroscope shows that the red substance is oxyhaemoglobin. This is very much more concentrated than the pigment in the blood. In a colourless population, too, a few individuals may have a pink shell gland, the imperceptible haemoglobin of the blood has accumulated there. The haemoglobin fills a part, or the whole, of the convoluted tubule. A microscope shows that the red substance is in the kidney's lumen, the cells of the wall appear free of it. A red shell gland is only occasionally found. Most populations are devoid of it and when it occurs it is usually found in less than 0·1 % of individuals. Exceptionally I have found it in nature in 2 % of *D. magna*.

Isolating individuals with a red gland shows that they are usually less viable than normal ones. It also shows that quite often the red colour disappears in the course of a day or two; the animals recover. I have never seen an individual with both shell glands affected, doubtless, as in man, at least one functional kidney is essential.

The impression made by the haemoglobinuria is that haemoglobin has accumulated in the shell gland owing to a blockage of the outlet. It suggests that perhaps haemoglobin as such is normally excreted in small quantities by the maxillary glands. However, affected animals in a red population are anaemic compared with normal ones, which seems to argue against this hypothesis.

(3) *A haemochromogen in the alimentary canal*

If a normal pink or red *D. magna* is examined under the microscope with a spectroscopic ocular, in a drop of water beneath the cover-slip of a compressorium, it shows two strong bands of oxyhaemoglobin in the blood. In a short time these bands fade and vanish, as the animal's respiration deoxygenates the haemoglobin The process can be much hastened by introducing a little sodium hydrosulphite solution beneath the cover-slip with a fine pipette. If the specimen is so arranged on the microscope stage that the field of vision under the high power is mostly filled by part of the anterior end of the midgut, then as the two oxyhaemoglobin bands fade a narrower band appears between them. This is first seen when the oxyhaemoglobin bands are half gone, and it becomes progressively stronger It is a narrow band like that of cytochrome *b* The wave-length of its axis, measured with the Zeiss spectroscopic ocular, calibrated with a neon lamp, is at $563\,m\mu$ A much weaker band can be seen at $533\,m\mu$. The pattern of absorption bands shows that the substance is a haemochromogen. If now (in the absence of hydrosulphite) the cover-slip is raised for a moment to admit air, these two bands vanish as the oxyhaemoglobin reappears. The haemochromogen thus reacts reversibly with dissolved oxygen in the oxidized form the strong bands go, when reduced again they reappear. For laboratory convenience I refer to this pigment as *daphniarubin* on the analogy of helicorubin, a haemochromogen in the crop liquid of snails which is reversibly oxidizable and reducible.

Helicorubin, in the crop liquid of the snail *Helix pomatia*, has a double α-band with axes at 563 and $558\,m\mu$, its β-band is at $532\,m\mu$. Clearly the two bands of daphniarubin correspond to the longer wave α-band of helicorubin and to its β-band. Exploration with the microspectroscope shows that daphniarubin is confined to the midgut, and to that part of it where there is a space between peritrophic membrane and wall, namely, in the front part of the midgut It is also in the anterior paired gut diverticula. In most individuals the pigment cannot be traced further back than opposite the heart, but occasionally it can be followed to the posterior bend of the intestine. In such cases the peritrophic membrane encloses little food and the gap between it and the gut wall can be seen to extend further back than usual. The bands of daphniarubin can be seen with the microspectroscope in the intestine

of *Daphnia* after it has been dissected out of the animal in a solution of sodium hydrosulphite. But as soon as the isolated intestine is torn and teased with needles the bands vanish. Clearly the pigment has leaked away. This shows that it is in solution in the liquid contents of the intestine, it is not situated in the gut wall. There is no daphniarubin in the eggs.

In helicorubin the reversible oxidation occurs only in an acid medium (Dhéré & Vegezzi 1917). When *Daphnia* is put for some hours into a dilute solution of bromothymol blue this indicator accumulates in the gut lumen, where it is much more concentrated than in the water outside. The mode of accumulation may be similar to that of chlorophyll discussed above. The indicator shows (without allowing for protein and other errors) that the anterior three-quarters of the midgut has a pH varying with individuals from 6 0 to 6·8, while the posterior quarter has 6·6 to 7·2. These figures agree sensibly with those found previously by von Dehn (1930) and Hasler (1935), they show that the front part of the gut of *Daphnia* has a suitable pH for the reversible oxidation of a pigment like helicorubin.

The amount of daphniarubin present in the intestine is proportional to the haemoglobin content of the animal's blood. Red *Daphnia* have most, pink ones have less. In pale or colourless individuals no daphniarubin can be detected in a single individual, though piling one on another may reveal it. The proportionality between daphniarubin and the blood haemoglobin suggests that the former may perhaps be the excretory product of the latter. Moreover, daphniarubin, with an α-band again at 563 mμ, is present also in the fairy shrimp *Chirocephalus diaphanus* Prévost, all along the gut; anaerobic conditions beneath a cover-slip reveal it through the microspectroscope. There must be less of the pigment here than in red *Daphnia* since the bands are no stronger although the animal is much bigger. *Chirocephalus* has haemoglobin in its blood (Lankester 1871), though the quantity is generally small.

There are, however, arguments against the suggestion that daphniarubin is the excretory product of haemoglobin in *Daphnia* and *Chirocephalus*. *Daphnia hyalina* Leydig is found in lake plankton; its blood is colourless. Heaping eggless individuals of this species on top of one another and examining them with the microspectroscope by transmitted light fails to show oxyhaemoglobin, but daphniarubin reveals itself after a few minutes of autoreduction. Here is daphniarubin without haemoglobin. Another objection is the fact that helicorubin is found in the snail's crop although the snail has no haemoglobin. But the snail's heart and buccal mass have cytochrome and there are haem compounds in liver, foot and elsewhere. The crayfish *Astacus pallipes* Lereboullet has a haemochromogen like daphniarubin in its hind gut liquid, with the α-band at 561 mμ. Here again there is no haemoglobin in the animal, but it has cytochrome, especially in the heart, and its liver contains haem. In the snail and crayfish the haemochromogen may represent the excretion of cytochrome and other haem compounds, in some water fleas and in the fairy shrimp perhaps that of haemoglobin.

(4) *Haematin in blood*

Under the heading of haemoglobin excretion one other point must be mentioned. Populations of *Daphnia magna* are sometimes found which are brown. This colour may be due to a greyish green tint of the eggs combined with an orange gut liquid and pink blood. But the brown aspect cannot always be accounted for thus. When *Daphnia* is squashed *en masse* the liquid, clarified by filtering through kieselguhr, is usually pink owing to haemoglobin, but occasionally it is brown. The intensity of the α-band of oxyhaemoglobin in such a filtrate from brown *D. magna* was found to match the intensity of the α-band in a much paler, and of course pinker, dilution of my own blood But the haemochromogen formed in the *Daphnia* filtrate by the addition of pyridine and sodium hydrosulphite was considerably more concentrated than that derived from my diluted blood Thus the brown colour is due, in part at least, to a haem compound which supplied the excess of pyridine haemochromogen.

This haem compound in the blood of brown *Daphnia* might be methaemoglobin or haematin. To test the first possibility, the filtered *Daphnia* extract was divided into two lots, to one of which sodium hydrosulphite was added If methaemoglobin were present it would thus be reduced to haemoglobin. Carbon monoxide was then passed through both lots. There was no increase either in pink colour or in the intensity of the absorption bands in the lot which had been treated with hydrosulphite, which showed that there had been no methaemoglobin there. It is therefore probable that the brown blood was coloured by haematin

This haematin is not necessarily an excretory product of the haemoglobin, but it is noteworthy as an alternative haem compound in the blood. Four months previously a particular brown population of *D magna* had been light pink, with a haemoglobin index of 42 So far as could be judged, the index of the brown populations was 25, but obviously this could not be accurately determined in view of the disturbing brown colour.

5. HAS THE HAEMOGLOBIN A FUNCTION?

(1) *The blood pigment is apparently functionless*

A low concentration of dissolved oxygen causes *Daphnia* to increase the haemoglobin content of its blood. The haemoglobin of *Ceriodaphnia laticaudata* has a higher oxygen affinity than that of *D. magna* (Fox 1945a), and the former lives in fouler water which is likely to contain less oxygen. These facts suggest that the blood pigment of the Cladocera is of use, and perhaps of vital importance.

When *Daphnia* is enclosed in a corked tube full of water the respiration of the animals gradually removes the dissolved oxygen from the water and after a time the haemoglobin in the blood becomes deoxygenated. The disappearance of the two absorption bands in the more or less crowded *Daphnia* population can be observed with a hand spectroscope. In one such experiment with *D. obtusa* the haemoglobin lost its oxygen in 20 min The animals continued swimming, however,

for a long time after this. An hour later many were actively swimming, and some were still swimming at the end of 5 hr. Such preliminary experiments showed that deoxygenation of the haemoglobin in the blood has no immediately fatal effect.

A series of experiments was next made to compare the activity and survival, in water containing little dissolved oxygen, of normal animals and of animals whose haemoglobin had been rendered functionless for oxygen transport by carbon monoxide. Ten experiments were made, in each of which twenty animals with carboxyhaemoglobin were enclosed in one stoppered bottle completely full of water, twenty untreated animals in another. The bottles had a capacity of 3 l., large enough for the respiration of the animals to have little effect on the concentration of dissolved oxygen. The initial oxygen content of the water was the same in each bottle; by previous bubbling with nitrogen it had been reduced to a value little above that at which the blood of *Daphnia* loses its oxygen (1·18 ml./l. at 17° C, see p. 197). The average oxygen concentration at the beginning of the experiments was 1·40 and at the end 1·13 ml./l. The experiments lasted 30 to 45 hr. Preliminary tests showed that the oxyhaemoglobin of *Daphnia* is rapidly converted into carboxy-haemoglobin by immersing the animals for less than 1 min. in aerated water 5 % saturated with carbon monoxide. This low proportion of carbon monoxide to oxygen should not affect cytochrome oxidase if present. The animals were left for 5 min. The water in the 3 l. bottle into which the treated animals were then put for the experiment was 1 % saturated with carbon monoxide to avoid dissociation of the carboxyhaemoglobin, and both this bottle and the control were kept in the dark. At the end of the experiment animals with carboxyhaemoglobin were examined with the microspectroscope in sodium hydrosulphite solution to see that there was no fading of the absorption bands in the blood. In these ten experiments there were 184 survivors of the 200 animals with carboxyhaemoglobin and 189 survivors of the 200 with oxyhaemoglobin. The difference is negligible and there was no visible difference in the activity of the two lots of survivors. The blood pigment appears thus to be unimportant in respiration. It may, of course, have some other function.

Artemia salina usually contains quite small amounts of haemoglobin. It is improbable that such low concentrations of blood pigment can be functional in respiration when the much more concentrated haemoglobin often present in *Daphnia magna* is apparently not so used. In *Artemia* the quantity of haemoglobin varies greatly in different individuals of a population. In some cases the oxyhaemo-globin bands can easily be seen in a single individual, in others the bands are faint or invisible. This variability argues against functional importance

(2) *Function of haemoglobin in parthenogenetic eggs*

If the blood pigment is functionless in respiration, is the haemoglobin in the eggs of use? The microspectroscope shows that the pond species *Daphnia magna*, *D. pulex* and *D. obtusa* have haemoglobin in the eggs within the brood pouch, even when the blood is more or less colourless. The lake plankton species *D. hyalina*, with

quite colourless blood, also has a trace of haemoglobin in the eggs. In this species the oxyhaemoglobin bands can be discerned with the spectroscopic ocular in a pile of parthenogenetic egg-bearing females heaped up moist on a microscopic slide, if examined quickly before the pigment is deoxygenated. A similar pile of eggless females shows no oxyhaemoglobin bands

Respiratory conditions are not ideal in the brood pouch This is clear from the following observations. If an individual of *D. magna, D. pulex* or *D. obtusa*, lightly held in a compressorium, is allowed to deoxygenate its haemoglobin by its own respiration, then the oxyhaemoglobin absorption bands fade first in the eggs and then in the blood. This might mean that the blood pigment has a higher affinity for oxygen than the haemoglobin of the eggs. But this is not so, for if the eggs are brought outside the brood pouch before the experiment begins by gently pressing on the brood pouch with a needle, then the oxyhaemoglobin bands in the eggs fade at the same time as those of the blood, or slightly later It may then be that, enclosed as they are in the brood pouch, the eggs need their haemoglobin for respiration during development.

The experiments with carboxyhaemoglobin described on p. 208 had a dual purpose. They were designed also to test the importance of the egg haemoglobin in development. The *D magna* used were parthenogenetic females with eggs, but not embryos, in the brood pouch. At the end of each experiment most individuals carried embryos, all of which were at the same stage of development in a given mother, but the stage reached in the different mothers varied, doubtless owing to initial differences in the degree to which cleavage had progressed Moreover, the experiments were purposely varied in duration from 30 to 45 hr. so that different embryonic stages should be attained. The stage reached was recorded for each mother at the end of an experiment. The following easily recognizable arbitrary stages were used· (1) uncleaved eggs, (2) cleaved eggs, (3) headless embryos, (4) embryos with head but no eyes, (5) two red eyes, antennae not free, (6) two red eyes, antennae free, (7) double black eye, (8) single black eye Table 1 groups together the number of mothers whose young reached the various developmental stages at the end of the ten experiments with and without carboxyhaemoglobin It is clear that up to stage 5 haemoglobin makes no difference in rate of development, but in the last three stages embryos without the functional respiratory pigment lag behind. χ^2 for the eight pairs of values is 60·25, giving a probability of less than 0·01 that the difference between the two series is due to chance.

Haemoglobin seems thus to have a function in embryonic development. Nevertheless carbon monoxide treated young show themselves to be just as active and

TABLE 1. NUMBERS OF *DAPHNIA MAGNA*, THE EMBRYOS OF WHICH
REACHED VARIOUS STAGES OF DEVELOPMENT

stage	1	2	3	4	5	6	7	8
with CO	2	10	15	76	33	38	6	4
without CO	1	14	18	71	38	14	19	13

viable as normal ones when kept for several days after hatching. Moreover, although the egg haemoglobin seems only to intervene in late embryonic stages, yet the quantity of haemoglobin in the egg diminishes as development proceeds This is best seen in *D. magna* and *D. pulex* with colourless blood and with so little haemoglobin in the eggs that the oxy-bands can only just be seen. As the embryos develop these bands disappear.

(3) *Absence of haemoglobin from ephippial eggs*

Considering the relatively large amount of haemoglobin in the parthenogenetic eggs, it is surprising to find no trace of the pigment in fertilized eggs taken out of the ephippium. This is so even with the most red blooded *D. magna*, *D. pulex* and *D obtusa*. These fertilized eggs, however, although lacking haemoglobin, contain abundant haem. This is shown by treating the eggs with pyridine and hydrosulphite, upon which a strong haemochromogen α-band appears.

The absence of haemoglobin in the fertilized eggs of *D. magna*, *D. pulex* and *D obtusa* and its presence in the parthenogenetic eggs of these species would seem to accord with the relatively better respiratory conditions in which the fertilized eggs can develop, since they are not enclosed in the brood pouch Yet this suggestion is not valid, for the fertilized eggs develop just as well at low as at high concentrations of dissolved oxygen. Experiments were made in which ephippia of *D. obtusa*, just freed from the mother, were put into three stoppered bottles completely filled with a relatively large volume of water having a dissolved oxygen content of 1·3 ml./l. at 18° C, i e. only 18 % saturated with air After 11 days the oxygen content of the water was then 1 2 ml /l. The young hatched at the same time as those out of other ephippia from the same population which had been kept in well-aerated water, namely on the 10th and 11th days

It might be suggested that the apparent lack of utility of haemoglobin in the blood of *Daphnia*, and its function, even if a minor one, in the development of parthenogenetic embryos, implies that the pigment in the blood is an overflow from a supply to the eggs. If this were so one might expect to find a different amount of blood pigment in parthenogenesis than in bisexual reproduction, since ephippial eggs lack the pigment. Yet in point of fact parthenogenetic females have no more blood haemoglobin than ephippial females in the same population.

At one period in the early history of animals haemoglobin must have appeared for the first time, a mere by-product of some metabolic chemical reaction. In mammals, in fishes and in *Daphnia* the production of haemoglobin is increased by oxygen deficit, perhaps this was a condition of its first appearance. Initially the haemoglobin would be useless. Then its potentially useful capacity for reversible oxygenation would in certain animals have been utilized. May it not be that in the blood of *Daphnia* haemoglobin still appears to-day as a mere by-product of semi-anaerobic metabolism?

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Mitotic activity in the adult male mouse, *Mus musculus* L. The diurnal cycles and their relation to waking and sleeping

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The diurnal cycles of mitotic activity in the ear epidermis of the adult male mouse have been determined by the removal of earclips at 2 hr intervals throughout the 24 hr The mice used were between 3 and 4 months old, and were of the Kreyberg white label and Strong's *CBA* strains A considerable degree of individual variation was found, but on the average the maximum mitotic activity was at 06 00 and 14 00 hr and the minimum mitotic activity at 10 00 and 20 00 hr

This observation was confirmed by killing groups of mice, each group consisting of five males, at the same 2 hr intervals throughout the 24 hr Similar variations in the mitotic activity of the ear epidermis were observed, and, in addition, similar cycles were evident in the mid-dorsal epidermis of the back, the stratified epithelium of the oesophagus, the lining epithelium of the epididymis, and the proliferating zone of the duodenal mucosa In this last tissue the rate of cell division never fell to a very low figure, and in the proliferating centres of the intestinal lymph nodules and in the seminiferous tubules of the testis there was no trace of a cycle since the rate of cell division remained constantly high

A study was also made of the spontaneous bodily activity of the mice throughout the 24 hr., and by comparing the average figures so obtained with the average figures for epidermal mitosis, it proved possible to make the significant correlation that when the animals are at rest mitotic activity is at a maximum and that when they are awake and active it is at a minimum

This correlation permits an explanation of the individual variation in mitotic activity, since there is also a high degree of individual variation in spontaneous bodily activity. It also permits an explanation of the contradictory results which have been reported in the past regarding diurnal mitosis rhythms in mice, since it is evident that the rhythms of bodily activity must be strongly affected by differences in the age, sex and condition of the animals used, in the season of the year, and in the routine of the laboratory.

It has recently been demonstrated (Bullough 1946) that mitotic activity in most of the tissues of the adult female mouse is cyclic, being related to the cyclic production of oestrogenic hormone by the ovaries, and further that this primary cycle has imposed upon it secondary cycles which are the results of the actions of two mitosis-controlling forces. In the case of the adult male mouse it is generally considered that the secretion of androgenic hormone by the testes is continuous and relatively steady. It therefore appeared to be of interest to attempt to discover how this fact influences mitosis, and whether it induces a pattern of activity differing from that seen in the female. Investigation of this point resulted in the discovery of strongly marked diurnal cycles of mitotic activity, and it quickly became evident that a thorough understanding of these was a necessary preliminary to any attempt to unravel conditions in the male. Particularly did this seem to be so when consideration was taken of the confusion and contradiction in the literature on diurnal mitosis cycles in the mouse and other animals. The present paper is therefore concerned with a detailed analysis of these diurnal cycles, especially in the epidermis, and of their relation to periods of waking and sleeping.

MATERIAL AND METHODS

(1) *The mice*

The male mice used in this work were almost all of the Kreyberg white label strain, but into each experimental group there was also inserted one male of the Strong's *CBA* strain. This was done to ensure that the results obtained were not peculiar to the type of mouse used, and, in fact, no differences between these strains were found. All the mice were in full breeding condition, and all were between 3 and 4 months of age when the experiments were performed. This was also true of the mice used in the experiments on bodily activity.

All the mice were in perfect health. They had been reared in a constant temperature of 20° C, and fed on whole oats, dog biscuit, and oat cake with cod-liver oil. These conditions were maintained during the experiments. In view of the fact that these experiments concern diurnal rhythms, it may also be important to add that the animals were accustomed to being fed, and were fed throughout the experiments, at between 09.00 and 10.00 hr., and that they had an almost constant daily period of natural and artificial light from about 08.00 hr. to about 18.00 hr.

(2) *Histological technique*

In the first experiments pieces of ear were cut from the mice by means of a conchotome. These were fixed for a day in Bouin's alcoholic fluid which also acted as a wetting agent for the skin and hair. They were then embedded in paraffin wax, and cut in serial sections 7μ thick. In the later experiments the animals used were killed with chloroform. They were then cut widely open along the midventral line,

dipped quickly in 70% alcohol as a wetting agent, and fixed in Bouin's ordinary fluid for 2 days. Tissues and organs removed were embedded in paraffin wax and cut at a thickness of 7μ. In all cases the mitoses were stained with Ehrlich's haematoxylin and the cytoplasm counterstained with eosin.

(3) *Statistical methods*

The methods of counting the mitoses varied according to the areas or lengths of section chosen as units. Information regarding these points is therefore given separately for each tissue in the observations recorded below. In the same way the method of assessing bodily activity throughout the 24 hr. is given later. When obtained, the figures for each experimental group were averaged, and the standard deviation was calculated according to the formula $\sigma = \sqrt{(\Sigma(fd^2)/N)}$, where f is the frequency, d the deviation from the mean, and N the number of mitosis counts in the group. In some groups there was considerable individual variation which resulted in a high standard deviation. In these circumstances the statistical significance of the differences observed was checked, and the results are described in the text. Each table includes data concerning the number of mitosis counts, the observed range of the variates, the arithmetic mean, and the standard error of the mean.

OBSERVATIONS

(1) *Mitotic activity of ear epidermis*

In any study of cyclic activity it is clearly a great advantage if the whole series of observations can be made on the same animals, and also if the experiments can be designed to inconvenience these animals as little as possible. The ear of the mouse is of great value for such study, since it can be cut away piece by piece without any great interference with normal activity. The operation was performed with a conchotome which in one movement punched out a piece of ear lobe of about 10 sq mm It was possible to obtain about six clips from each ear, and thus from each animal twelve samples could be taken The clips were removed at 2 hr. intervals throughout the 24 hr , the experiment being started at 10 00 hr on one day and concluded at 08.00 hr. on the next. To avoid any chance of the development of a high rate of mitosis due to wounding, each new clip cut away the tissue immediately adjacent to the gap left by the previous clip, and the results showed that in 2 hr. there was no time for any wounding effect to develop.

The mitosis counts were made on 1 cm. lengths of sections of the epidermis cut at 7μ, and for each clip it was found possible to make ten such counts with ease. To avoid the danger of counting the same mitosis twice, every alternate section of the series was ignored. The mitoses were found most commonly in the metaphase, but all stages from prophase to telophase were noticed and recorded. The average figures obtained for all the ten mice studied are given in table 11 and illustrated in graph 11, figure 3, but in view of the high degree of individual variation, especially at night, the results for each mouse are also given separately in tables 1 to 10 and

TABLE 1. NUMBERS OF MITOSES PRESENT IN SECTIONS OF THE EAR
EPIDERMIS OF MOUSE 37 (SEE ALSO GRAPH 1, FIGURE 1)

time of day (G.M T.)	number of mitoses in 10 cm.					number of mitoses per cm.		
	pro-phase	meta-phase	ana-phase	telo-phase	total	number	range	mean ± standard error
10.00	1	20	2	8	31	10	2–6	3·1 ± 0·38
12.00	1	57	15	13	86	10	6–14	8·6 ± 0·86
14.00	2	44	8	27	81	10	6–11	8·1 ± 0·48
16.00	3	17	4	14	38	10	2–5	3·8 ± 0·38
18.00	2	36	3	7	48	10	3·7	4 8 ± 0·35
20.00	0	0	0	15	15	10	0–3	1·5 ± 0·25
22.00	0	2	0	11	13	10	0–3	1·3 ± 0·22
24 00	4	35	2	6	47	10	3–7	4·7 ± 0·35
02.00	2	33	0	18	53	10	4–7	5·3 ± 0·35
04.00	0	0	2	35	37	10	2–5	3·7 ± 0 25
06.00	1	30	1	12	44	10	3–6	4 4 ± 0 25
08.00	0	62	0	10	72	10	6–9	7·2 ± 0·35

TABLE 2. NUMBERS OF MITOSES PRESENT IN SECTIONS OF THE EAR
EPIDERMIS OF MOUSE 38 (SEE ALSO GRAPH 2, FIGURE 1)

time of day (G.M.T.)	number of mitoses in 10 cm.					number of mitoses per cm.		
	pro-phase	meta-phase	ana-phase	telo-phase	total	number	range	mean ± standard error
10 00	0	0	0	3	3	10	0–1	0 3 ± 0 03
12 00	1	6	0	1	8	10	0–2	0·8 ± 0·19
14.00	0	15	0	11	26	10	1 4	2 6 ± 0 29
16.00	0	56	3	20	79	10	5–12	7 9 ± 0·63
18 00	0	48	4	34	86	10	6–10	8 6 ± 0·41
20 00	1	18	1	1	21	10	0–4	2·1 ± 0·32
22 00	0	0	0	8	8	10	0–2	0 8 ± 0·22
24 00	1	22	0	0	23	10	1–4	2 3 ± 0 29
02 00	0	0	0	17	17	10	1–3	1·7 ± 0 25
04.00	0	23	2	3	28	10	1–5	2 8 ± 0 35
06.00	1	43	2	4	50	10	4–6	5·0 ± 0 29
08 00	0	28	3	16	47	10	3–6	4·7 ± 0·32

TABLE 3. NUMBERS OF MITOSES PRESENT IN SECTIONS OF THE EAR
EPIDERMIS OF MOUSE 39 (SEE ALSO GRAPH 3, FIGURE 1)

time of day (G.M.T.)	number of mitoses in 10 cm.					number of mitoses per cm		
	pro-phase	meta-phase	ana-phase	telo-phase	total	number	range	mean ± standard error
10.00	1	2	0	18	21	10	1–3	2 1 ± 0 25
12.00	0	65	2	17	84	10	7–11	8·4 ± 0·41
14.00	0	42	2	19	63	10	4 8	6 3 ± 0·35
16 00	0	12	1	16	29	10	1–4	2 9 ± 0 29
18 00	0	35	5	14	54	10	4–7	5·4 ± 0·38
20.00	0	9	0	4	13	10	0–2	1 3 ± 0·19
22.00	0	4	0	5	9	10	0–2	0 9 ± 0·22
24 00	1	51	0	8	60	10	5–8	6·0 ± 0·32
02 00	0	2	0	6	8	10	0–2	0·8 ± 0·19
04.00	1	32	5	33	71	10	5–10	7·1 ± 0·44
06.00	0	110	3	9	122	10	9–15	12·2 ± 0·57
08.00	0	43	1	15	59	10	5–7	5·9 ± 0·25

TABLE 4. NUMBERS OF MITOSES PRESENT IN SECTIONS OF THE EAR
EPIDERMIS OF MOUSE 40 (SEE ALSO GRAPH 4, FIGURE 1)

time of day (G.M.T.)	number of mitoses in 10 cm.					number of mitoses per cm.		
	pro-phase	meta-phase	ana-phase	telo-phase	total	number	range	mean ± standard error
10.00	0	0	0	9	9	10	0–2	0·9 ± 0·06
12.00	1	35	1	1	38	10	2–5	3·8 ± 0·29
14.00	0	48	2	17	67	10	5–8	6·7 ± 0·35
16.00	0	35	6	29	70	10	5–10	7·0 ± 0·48
18 00	0	26	2	16	44	10	3–6	4·4 ± 0·29
20.00	0	3	0	18	21	10	1–3	2 1 ± 0·16
22.00	0	18	0	2	20	10	0–3	2·0 ± 0·32
24.00	0	2	0	3	5	10	0–1	0·5 ± 0·16
02.00	0	14	0	0	14	10	C -3	1·4 ± 0·25
04.00	3	16	3	13	35	10	1–5	3·5 ± 0·38
06 00	2	90	3	7	102	10	8–13	10 2 ± 0·48
08 00	0	31	0	7	38	10	3–5	3 8 ± 0·29

TABLE 5. NUMBERS OF MITOSES PRESENT IN SECTIONS OF THE EAR
EPIDERMIS OF MOUSE 41 (SEE ALSO GRAPH 5, FIGURE 1)

time of day (G.M.T.)	number of mitoses in 10 cm.					number of mitoses per cm.		
	pro-phase	meta-phase	ana-phase	telo-phase	total	number	range	mean ± standard error
10.00	0	0	0	4	4	10	0–1	0 4 ± 0·16
12.00	—	—	—	—	0	10	- –	0
14.00	0	44	7	7	58	10	4 8	5·8 ± 0·41
16.00	2	1	0	20	23	10	1–4	2 3 ± 0·29
18.00	0	14	1	2	17	10	1–3	1·7 ± 0·25
20.00	0	2	0	10	12	10	0–2	1 2 ± 0·19
22.00	0	4	0	0	4	10	0–1	0·4 ± 0·16
24.00	1	65	2	4	72	10	6–9	7·2 ± 0 32
02.00	0	6	0	25	31	10	2–4	3 1 ± 0·25
04.00	2	134	5	3	144	10	11–17	14·4 ± 0·66
06.00	3	28	0	9	40	10	2–5	4 0 ± 0 32
08 00	0	11	4	12	27	10	1 -4	2 7 ± 0·35

TABLE 6. NUMBERS OF MITOSES PRESENT IN SECTIONS OF THE EAR
EPIDERMIS OF MOUSE 42 (SEE ALSO GRAPH 6, FIGURE 2)

time of day (G M T)	number of mitoses in 10 cm.					number of mitoses per cm.		
	pro-phase	meta-phase	ana-phase	telo-phase	total	number	range	mean ± standard error
10.00	4	18	0	10	32	10	2–5	3·2 ± 0·32
12.00	7	81	2	13	103	10	7–14	10·3 ± 0·66
14 00	3	26	3	39	71	10	5–10	7·1 ± 0·57
16.00	1	47	0	9	57	10	4–7	5·7 ± 0·29
18.00	1	53	12	58	124	10	9–16	12 4 ± 0 66
20.00	0	3	0	10	13	10	0–2	1 3 ± 0·19
22.00	2	57	2	4	65	10	5–9	6·5 ± 0·38
24.00	3	1	0	24	28	10	1–4	2·8 ± 0·32
02.00	5	37	0	0	42	10	3–5	4·2 ± 0·25
04.00	4	22	5	16	47	10	3–7	4 7 ± 0·38
06 00	1	141	3	39	184	10	14–22	18·4 ± 0·73
08.00	0	11	4	31	46	10	3–6	4·6 ± 0·29

TABLE 7. NUMBERS OF MITOSES PRESENT IN SECTIONS OF THE EAR
EPIDERMIS OF MOUSE 43 (SEE ALSO GRAPH 7, FIGURE 2)

time of day (G.M.T.)	number of mitoses in 10 cm.					number of mitoses per cm.		
	pro-phase	meta-phase	ana-phase	telo-phase	total	number	range	mean ± standard error
10.00	3	64	2	8	77	10	7–11	7·7 ± 0·44
12.00	9	34	0	9	52	10	4–6	5·2 ± 0·10
14.00	1	41	2	24	68	10	5–8	6·8 ± 0·41
16.00	3	80	0	26	109	10	8–14	10·9 ± 0·60
18.00	0	21	4	25	50	10	4–6	5·0 ± 0·06
20.00	1	39	1	6	47	10	3–7	4·7 ± 0·38
22.00	0	43	7	28	78	10	5–10	7·8 ± 0·48
24.00	0	17	4	16	37	10	3–5	3·7 ± 0·13
02.00	1	63	0	18	82	10	5–11	8·2 ± 0·19
04.00	0	8	0	7	15	10	0–2	1·5 ± 0·19
06·00	0	46	1	3	49	10	3–7	4·9 ± 0·41
08.00	0	12	2	14	28	10	1–4	2·8 ± 0·32

TABLE 8. NUMBERS OF MITOSES PRESENT IN SECTIONS OF THE EAR
EPIDERMIS OF MOUSE 44 (SEE ALSO GRAPH 8, FIGURE 2)

time of day (G.M.T)	number of mitoses in 10 cm.					number of mitoses per cm.		
	pro-phase	meta-phase	ana-phase	telo-phase	total	number	range	mean ± standard error
10.00	0	23	2	22	47	10	3–7	4·7 ± 0·38
12.00	2	17	0	0	19	10	1–3	1·9 ± 0·22
14 00	3	46	9	84	142	10	11–18	14·2 ± 0·57
16.00	6	78	7	14	105	10	9–13	10·5 ± 0·44
18.00	0	25	8	58	91	10	6–12	9·1 ± 0·60
20 00	1	2	0	0	3	10	0–1	0·3 ± 0·13
22 00	0	39	2	25	66	10	5–9	6·6 ± 0·41
24.00	0	41	0	10	51	10	4·6	5·1 ± 0·25
02 00	0	12	0	4	16	10	1 3	1·6 ± 0·25
04.00	3	25	0	0	28	10	1–5	2·8 ± 0·35
06.00	1	41	5	52	99	10	8–13	9·9 ± 0·51
08.00	3	25	0	14	42	10	3–6	4·2 ± 0·29

TABLE 9. NUMBERS OF MITOSES PRESENT IN SECTIONS OF THE EAR
EPIDERMIS OF MOUSE 45 (SEE ALSO GRAPH 9, FIGURE 2)

time of day (G.M.T.)	number of mitoses in 10 cm.					number of mitoses per cm.		
	pro-phase	meta-phase	ana-phase	telo-phase	total	number	range	mean ± standard error
10.00	1	13	2	11	27	10	1–4	2·7 ± 0·29
12.00	12	44	0	0	56	10	4–7	5·6 ± 0·32
14.00	4	43	8	16	71	10	6 9	7·1 ± 0·35
16.00	3	32	9	21	65	10	5–8	6·5 ± 0·35
18.00	0	9	0	9	18	10	0–3	1·8 ± 0·29
20.00	2	4	0	1	7	10	0–2	0·7 ± 0·19
22.00	1	14	4	21	40	10	2–6	4·0 ± 0·35
24.00	0	47	7	32	86	10	7–11	8·6 ± 0·18
02 00	0	14	0	10	24	10	1–3	2·4 ± 0·19
04·00	0	0	0	1	1	10	0–1	0·1 ± 0·10
06·00	2	68	7	41	118	10	9–14	11·8 ± 0·48
08 00	1	33	0	29	63	10	5–8	6·3 ± 0·41

TABLE 10. NUMBERS OF MITOSES PRESENT IN SECTIONS OF THE EAR
EPIDERMIS OF MOUSE 46 (SEE ALSO GRAPH 10, FIGURE 2)

time of day (G.M T.)	number of mitoses in 10 cm.					number of mitoses per cm.		
	pro-phase	meta-phase	ana-phase	telo-phase	total	number	range	mean ± standard error
10.00	5	14	2	49	70	10	6–9	7·0 ± 0·32
12.00	2	72	5	13	92	10	7–12	9·2 ± 0·48
14.00	1	59	5	32	97	10	7–12	9·7 ± 0·51
16.00	1	40	2	21	64	10	4–8	6·4 ± 0·41
18.00	0	23	3	29	55	10	4–7	5·5 ± 0·38
20.00	0	5	0	6	11	10	0–3	1·1 ± 0·29
22.00	0	27	4	47	78	10	6–10	7·8 ± 0·35
24.00	0	18	2	4	24	10	1–4	2·4 ± 0·29
02.00	2	36	3	16	57	10	4–7	5·7 ± 0·32
04.00	1	7	0	4	12	10	0–2	1·2 ± 0·19
06.00	0	52	3	40	95	10	8–12	9·5 ± 0·41
08 00	3	36	2	43	84	10	7–11	8·4 ± 0·41
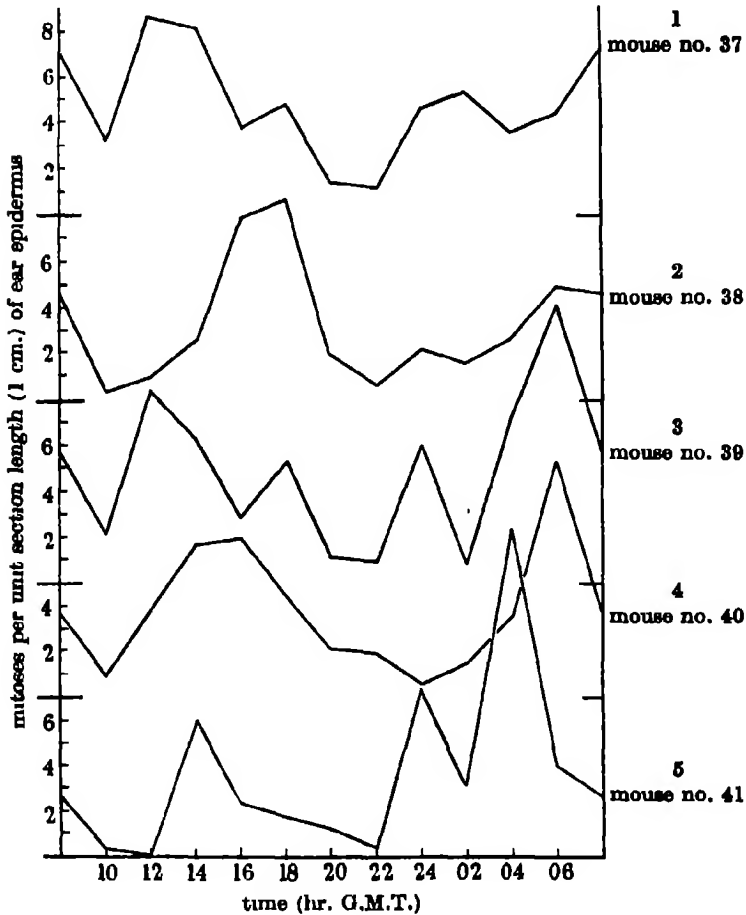
FIGURE 1. Graphs 1 to 5 indicating the changes in the mitotic activity of the ear
epidermis of individual male mice during a period of 24 hr.

in graphs 1 to 10, figures 1 to 2. In these tables the numbers of cell divisions in the pro-, meta-, ana- and telophases are also shown as they help to illustrate the passage of each mitosis wave.
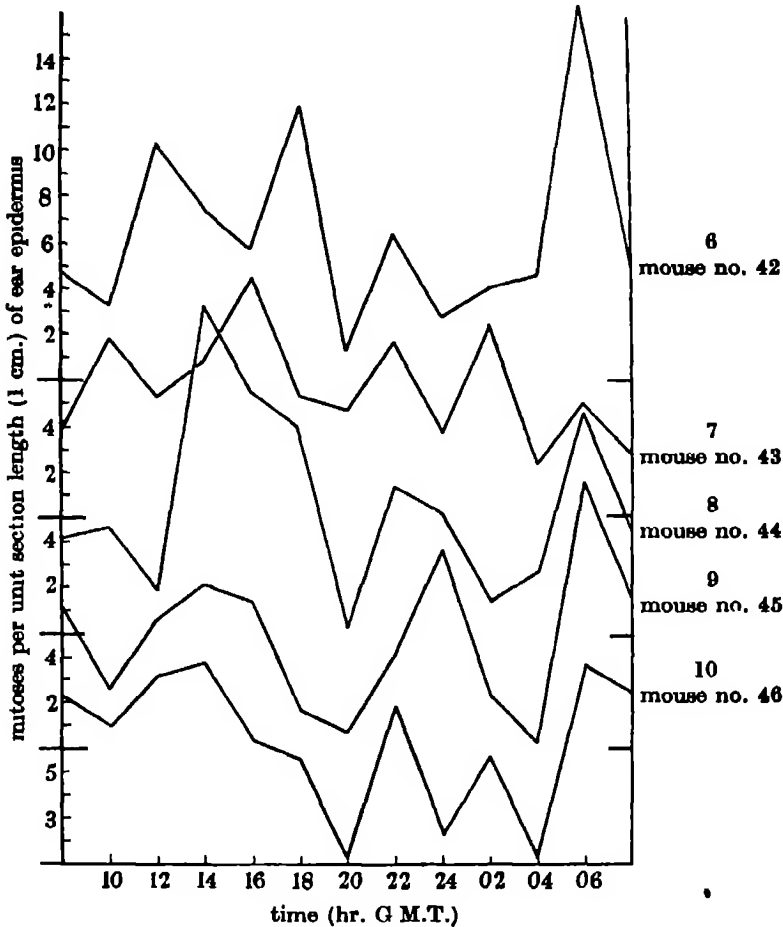
FIGURE 2. Graphs 6 to 10 indicating the changes in the mitotic activity of the ear epidermis of individual male mice during a period of 24 hr.

In spite of the considerable individual variation shown in the above tables, it can, nevertheless, be seen that there is some measure of agreement. Thus it is common for mitotic activity to be low at about 10.00 hr., high during the afternoon, low again at about 20.00 or 22.00 hr., and high again in the early morning. Individual irregularity is most marked during the night, and there is an evident tendency for mitotic activity to proceed in sharp waves. This latter effect is most clearly seen in the figures for the pro- and metaphases, that is, in the figures which show the numbers of mitoses beginning.

Individual variations are eliminated when all the above figures are averaged. These averages are given in table 11 and illustrated in graph 11, figure 3, and they show clearly the existence of a double diurnal cycle. The times of lowest average mitotic activity are 10.00 and 20.00 hr., while the times of highest average mitotic activity are 14.00 and 06.00 hr. There is also a long period of moderate mitotic activity lasting from 22.00 to 04.00 hr.

TABLE 11. AVERAGE NUMBERS OF MITOSES PRESENT PER UNIT LENGTH (1 CM.) OF THE EAR EPIDERMIS OF MICE 37 TO 46 (SEE ALSO GRAPH 11, FIGURE 3)

time of day (G.M.T.)	number of counts	range	mean ± standard error
10.00	100	0–11	3·2 ± 0·24
12.00	100	0–14	5·4 ± 0·10
14.00	100	1–18	7·4 ± 0·09
16.00	100	1–14	6·4 ± 0·09
18.00	100	0–16	5·9 ± 0·10
20.00	100	0–7	1·6 ± 0·04
22.00	100	0–10	3·8 ± 0·10
24.00	100	0–11	4·3 ± 0·07
02.00	100	0–11	3·4 ± 0·07
04.00	100	0–17	4·2 ± 0·12
06.00	100	2–22	9·0 ± 0·14
08.00	100	1–11	5·1 ± 0·06
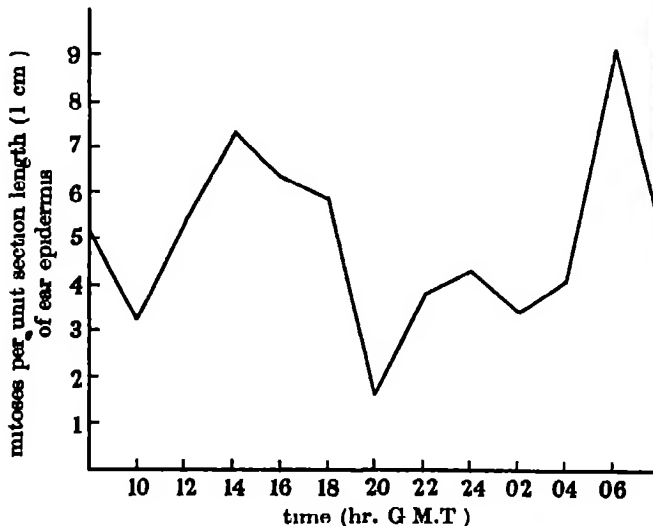
FIGURE 3. Graph 11 showing the average numbers of mitoses in the ear epidermis of the ten mice 37 to 46 during a period of 24 hr.

As a check on this result, the experiment was repeated at a later date using another ten mice. In view of the similarity of the findings it does not appear necessary to repeat the individual results in full, but table 12 summarizes the average results.

TABLE 12. CONFIRMATORY EXPERIMENT SHOWING THE AVERAGE NUMBERS OF
MITOSES PRESENT PER UNIT LENGTH (1 CM.) OF THE EAR EPIDERMIS OF TEN
MALE MICE

time of day (B.S.T.)	number of counts	range	mean ± standard error
10.00	100	0–7	2 7 ± 0·15
12.00	100	1 9	5·1 ± 0 17
14.00	100	2–18	8·9 ± 0 10
16.00	100	1- 16	7·1 ± 0·11
18.00	100	1–16	5 4 ± 0 12
20.00	100	0 5	2 0 ± 0 04
22.00	100	0–7	3·8 ± 0·07
24.00	100	0–10	4·3 ± 0·06
02 00	100	1–9	4·7 ± 0 09
04.00	100	1–11	5·2 ± 0·16
06 00	100	3–19	9 3 ± 0 15
08 00	100	2 -13	6·5 ± 0·11

An interesting feature of these results is that they were obtained after the changing of the clock from G M.T. to summer time, and that in spite of this, the usual maxima occur at 06.00 and 14.00 hr. and the usual minima at 10 00 and 20.00 hr. as reckoned by the new time. It is evident that the mice had adapted themselves to the new conditions, presumably by accustoming themselves to the change in the habits of the staff which cared for them and particularly to the change in the feeding time.

(2) *Mitotic activity of other tissues*

In no tissue other than the ear epidermis is the accurate study of variations in mitotic activity easily possible, since no other tissue can be removed at frequent intervals with so little disturbance to the daily routine of the mouse However, it is obviously of considerable importance to determine whether the mitotic activity of the other tissues proceeds in such clearly marked cycles, and if so, whether the timing of these cycles is similar or different. To obtain answers to these questions it was necessary to kill groups of mice through the 24 hr., each group consisting of five males. The first such group was chloroformed at 10 00 hr., and the others followed at 2 hr. intervals until the last was killed at 08 00 hr on the succeeding day. Thus a total of sixty mice was involved.

The choice of tissues which could be removed and analyzed was limited by the fact that the numbers of mitoses in so many of them were too low for accurate counting Those described below were chosen to present as wide a variety of types as possible. As a preliminary, it was decided to repeat once again the observations on the ear epidermis, partly as a check on the results recorded above, but mainly as a check on the reliability of this method of studying mice killed in successive groups.

(i) *Ear epidermis*

In this experiment the earclips were taken after the mice had been killed and fixed, and for each animal 10 cm. lengths of sections cut 7μ thick were examined.

Thus fifty counts were made for each group of five animals. It seemed that no useful purpose would be served by classifying the stages of the mitoses, and therefore in the figures given in table 13 all stages from prophase to telophase are included.

TABLE 13. AVERAGE NUMBERS OF MITOSES PRESENT PER UNIT LENGTH (1 CM.) IN SECTIONS OF EAR EPIDERMIS (SEE ALSO GRAPH 13, FIGURE 4)

time of day (G.M.T.)	number of counts	range	mean ± standard error
10.00	50	0–5	2·4 ± 0·17
12.00	50	0–8	2·8 ± 0·28
14.00	50	5–15	8·3 ± 0·31
16.00	50	1–8	•·6 ± 0·28
18.00	50	1–9	4·4 ± 0·30
20.00	50	1–10	4·2 ± 0·27
22.00	50	0–4	1·9 ± 0·16
24.00	50	0–5	2·2 ± 0·18
02.00	50	4–13	7·5 ± 0·31
04.00	50	3–13	7·0 ± 0·40
06.00	50	5–14	8·7 ± 0·31
08.00	50	2–8	5·2 ± 0·23

These figures are expressed in graph 13, figure 4, and a comparison of this with graph 11 shows that, although differences in detail do exist, there are nevertheless broad similarities. Thus in graph 13 the times of lowest activity are 10.00 and 22.00 hr., while in graph 11 they are 10.00 and 20.00 hr. In both graphs the times of highest activity are 14.00 and 06.00 hr. It may be concluded that while the method of killing mice in successive groups is undoubtedly not so accurate as that of removing successive earclips, it is sufficiently accurate to reveal the main outlines of any cycles of mitotic activity which may exist.

(ii) Mid-dorsal epidermis

The second tissue studied was also epidermis, but in this case it came from the middle of the back. This was done in order to discover whether the variations in the ear epidermis can be considered as representative of the variations in the epidermis as a whole. As before, the sections were cut at 7μ, and the numbers of mitoses were counted in unit section lengths of 1 cm. The results are shown in table 14 and in graph 12, figure 4. While the figures are higher than those for the ear epidermis, the variations are almost identical with minima at 10.00 and 22.00 hr. and maxima at 14.00 and 06.00 hr.

(iii) Oesophagus

Sections were cut transversely in that region of the oesophagus just anterior to the diaphragm, and the stratified lining epithelium was found to show a sufficiently high rate of mitotic activity for accurate counts to be made. Again the method of counting was the same with unit section lengths of 1 cm., with ten counts for each

TABLE 14. AVERAGE NUMBERS OF MITOSES PRESENT PER UNIT LENGTH (1 CM.)
IN SECTIONS OF MID-DORSAL EPIDERMIS (SEE ALSO GRAPH 12, FIGURE 4)

time of day (G.M.T.)	number of counts	range	mean ± standard error
10.00	50	2–8	5·3 ± 0·30
12.00	50	4–15	8·6 ± 0·34
14.00	50	13–23	17·6 ± 0·54
16.00	50	10–20	14·7 ± 0·44
18.00	50	9–15	11·8 ± 0 35
20.00	50	8–13	10·8 ± 0·37
22.00	50	3–8	5·0 ± 0·21
24.00	50	3–8	5·9 ± 0·23
02.00	50	8–15	11·2 ± 0·28
04 00	50	7–17	12·6 ± 0·61
06.00	50	13–25	18·5 ± 0 68
08.00	50	10–16	13·8 ± 0 37
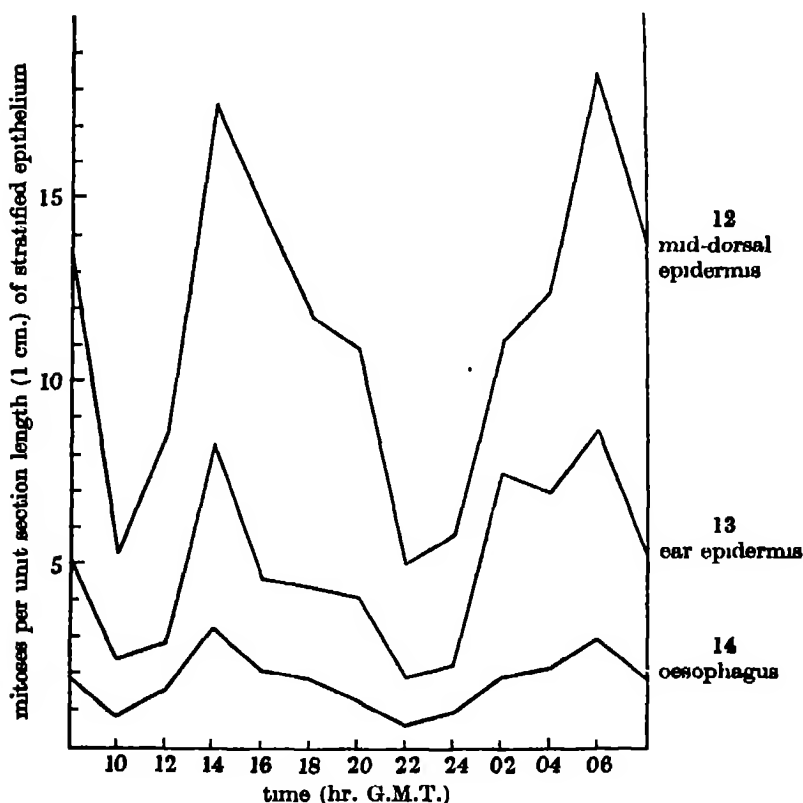
FIGURE 4. Graphs 12 to 14 showing the variations in the average numbers of mitoses in the stratified epithelia of the mid-dorsal epidermis, the ear, and the oesophagus.

animal, and with fifty counts for each group. The results are shown in table 15 and graph 14. Here again, in spite of the fact that the mitotic activity of the stratified epithelium was considerably less than that of the ear epidermis, the cyclic variations were similar with the usual minima at 10.00 and 22.00 hr. and the usual maxima at 14.00 and 06 00 hr.

TABLE 15. AVERAGE NUMBERS OF MITOSES PRESENT PER UNIT LENGTH (1 CM.) IN SECTIONS OF THE STRATIFIED EPITHELIUM OF THE OESOPHAGUS (SEE ALSO GRAPH 14, FIGURE 4)

time of day (G.M.T.)	number of counts	range	mean ± standard error
10.00	50	0–2	0·8 ± 0·07
12.00	50	0–3	1·5 ± 0·10
14.00	50	2–5	3·2 ± 0·13
16.00	50	1–4	2·1 ± 0·10
18.00	50	1–3	1 8 ± 0·09
20.00	50	0–3	1·4 ± 0·10
22.00	50	0–2	0·7 ± 0 07
24.00	50	0–2	1·0 ± 0·09
02.00	50	0–3	1·9 ± 0·10
04.00	50	1–4	2·2 ± 0·11
06·00	50	2–4	3·0 ± 0·10
08 00	50	1–3	1 9 ± 0·09

(iv) *Epididymis*

This was chosen as a representative of the accessory sexual organs, and the mitoses were counted in the lining epithelium of the tubules. The counting was done in that region in which the lining cells are of a particularly tall columnar form. In these cells the resting nuclei are withdrawn to the cell bases, and the mitoses are unusually prominent since, when they are about to divide, the nuclei move upwards into the clear middle region of the cell. Their prominence is even

TABLE 16. AVERAGE NUMBERS OF MITOSES PRESENT PER UNIT AREA (0·1 SQ.MM.) IN SECTIONS OF THE EPIDIDYMIS (SEE ALSO GRAPH 15, FIGURE 5)

time of day (G.M.T.)	number of counts	range	mean ± standard error
10.00	100	0–1	0·12 ± 0 032
12.00	100	0–2	0·36 ± 0·059
14 00	100	0–4	0·97 ± 0·112
16.00	100	0–2	0·51 ± 0·062
18.00	100	0–2	0·33 ± 0·050
20.00	100	0–1	0·14 ± 0·035
22 00	100	0–1	0·08 ± 0·027
24.00	100	0–2	0·37 ± 0·052
02.00	100	0–3	0·31 ± 0·058
04.00	100	0–3	0·64 ± 0·067
06.00	100	0–2	0·29 ± 0·047
08.00	100	0–1	0·16 ± 0·032

further enhanced by the exceptionally large size of the mitotic figures. It may be noted that in the other regions of the epididymis, where the cells of the lining epithelium are more nearly cubical, mitoses do not appear to be so frequent.

For the practical purpose of counting the numbers of cell divisions, the tissue of the epididymis was regarded as homogenous. The numbers of mitoses were assessed in unit section areas of 0·1 sq.mm., and the figures obtained are recorded in table 15 and graph 15, figure 5. Because of the low numbers of mitoses present, twenty counts were made for each animal and 100 for each group. In this tissue the rate of cell division was relatively low, and since so many of the unit areas examined contained no mitoses, the standard errors are unusually high. This means that, although the graph follows the normal trend almost exactly with minima at 10.00 and 22.00 hr. and maxima at 14.00 and 04.00 hr., the degree of difference between any two adjacent points is not statistically significant. However, since the differences between the maxima and the minima are statistically highly significant, it may be concluded that here again the cycle of variation is similar to that of the ear epidermis.

(v) *Duodenal mucosa*

Transverse sections of the duodenum were cut at a level of about 3 cm. from the pyloric sphincter, and mitoses were found to be most frequent in the cubical cells lining the crypts of Lieberkuhn These crypts are tortuously twisted and closely packed together so that it was possible to treat them as if they were a solid and homogeneous mass of tissue. Counts were therefore made on unit section areas of 0·1 sq.mm. in this proliferating region, and the figures obtained are presented in table 17 and graph 16, figure 5. Ten counts were made on each animal and fifty on each group. It is evident that in this tissue the rate of cell division continues to be relatively high throughout the whole 24 hr. However, there are clearly two minima at 10.00 and·22.00 hr. and one maximum at 14.00 hr. There is no sharply defined

TABLE 17. AVERAGE NUMBERS OF MITOSES PRESENT PER UNIT AREA (0·1 SQ.MM.)
OF SECTIONS OF THE PROLIFERATING ZONE OF THE DUODENAL MUCOSA (SEE
ALSO GRAPH 16, FIGURE 5)

time of day (G M T)	number of counts	range	mean ± standard error
10.00	50	5–14	9 5 ± 0 41
12.00	50	6–20	12·2 ± 0 59
14.00	50	10–27	16·5 ± 0 52
16.00	50	6–17	10·8 ± 0·47
18.00	50	5–18	12·3 ± 0·54
20.00	50	6–21	12 2 ± 0·59
22.00	50	6–18	9·8 ± 0·44
24.00	50	7–24	16·4 ± 0·58
02.00	50	8–24	16 5 ± 0·58
04.00	50	7–21	14·1 ± 0·52
06.00	50	9–24	15·8 ± 0·43
08.00	50	7–19	13·7 ± 0·48

second maximum in the early hours of the morning, but instead the rate of division continues to be almost uniformly high from about midnight to 06.00 or 08.00 hr. The graph is flatter than that for any tissue so far described, which is to say that the differences between the minima and the maxima are not so great.

(vi) *Intestinal lymph nodes*

As is known from previous work on the female mouse (Bullough 1946), the lymph nodes of the intestinal wall exhibit special conditions of mitotic activity. There is a high non-cyclic rate of division among the large cells which compose their centres, and a total absence of mitosis among the smaller lymphocytes which form their outer zones. The results of counts made on unit section areas of 0·1 sq.mm. of the proliferating centres are given in table 18 and graph 17, figure 5. As in the female, the rate of cell division in the lymph node centres is seen to be constantly high, and so to present an exception to the general rule. Statistically the slight differences recorded between the different groups of figures are not significant, and the graph may therefore be considered as approximating to a straight line.

TABLE 18. AVERAGE NUMBERS OF MITOSES PRESENT PER UNIT AREA (0·1 SQ MM.) OF SECTIONS OF THE PROLIFERATING CENTRES OF THE INTESTINAL LYMPH NODES (SEE ALSO GRAPH 17, FIGURE 5)

time of day (G.M.T.)	number of counts	range	mean ± standard error
10.00	50	9–20	13·9 ± 0 45
12 00	50	8–18	12 4 ± 0 41
14.00	50	10–20	14 0 ± 0·41
16.00	50	8–18	13·0 ± 0·34
18 00	50	8–19	12 7 ± 0·37
20 00	50	7–19	13 1 ± 0·41
22.00	50	7–18	12·7 ± 0 43
24.00	50	9–18	13·1 ± 0 34
02 00	50	8–20	12 9 ± 0·47
04 00	50	9–19	14 0 ± 0·40
06.00	50	8–18	12·1 ± 0·43
08.00	50	7–18	11 4 ± 0·44

(vii) *Testis*

It is well known that cell division within the testis proceeds in what are known as spermatogenic waves, each of which passes slowly along the length of a semi-niferous tubule. However, it was quickly apparent that the initiation and move-ment of these waves are in no way related to any diurnal rhythm. It was also evident from only a superficial examination of the seminiferous tubules that the rate of cell division is extremely high. To test for the existence of any diurnal rhythm, counts of mitoses and meioses were made in unit section areas of 0·01 sq.mm. The results are given in table 19 and in graph 18, figure 5. From these figures it is clear that cell division in the seminiferous tubules continues at a high rate both day and night as is the case in the lymph node centres.

TABLE 19. AVERAGE NUMBERS OF MITOSES PRESENT PER UNIT AREA (0·01 SQ MM.)
OF SECTIONS OF THE SEMINIFEROUS TUBULES (SEE ALSO GRAPH 18, FIGURE 5)

time of day (G.M.T.)	number of counts	range	mean ± standard error
10.00	50	12–26	19·7 ± 0·41
12.00	50	13–25	20·6 ± 0·40
14 00	50	13–28	20·1 ± 0 45
16 00	50	12–26	19 8 ± 0 44
18.00	50	11–26	19 0 ± 0 43
20.00	50	12–24	18·6 ± 0 26
22 00	50	11 –26	19·5 ± 0·51
24.00	50	12–25	19·6 ± 0·52
02.00	50	13–27	20·1 ± 0·44
04.00	50	12–27	20·3 ± 0·50
06.00	50	12–31	22·4 ± 0·74
08 00	50	13–28	20 8 ± 0·48
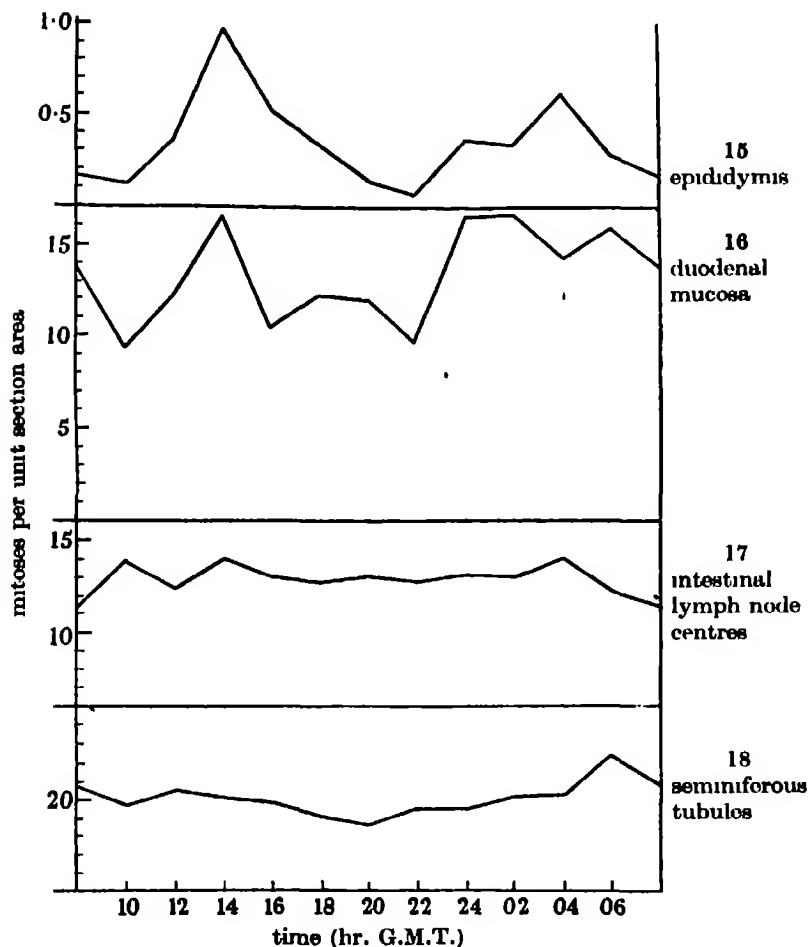
FIGURE 5. Graphs 15 to 18 showing the variations in the average numbers of mitoses in the epididymis, the duodenal mucosa, the intestinal lymph node centres, and the seminiferous tubules of the testis.

(3) *Bodily activity*

From a consideration of the above results, the question arises whether the cycles observed are due to variations in metabolic rate, body temperature, or some other factor which is affected by the alternation between activity and rest. Clearly the first task was to discover as precisely as possible at what times of the day and night the mice were active, and at what times they were at rest. The habits of any colony of mice are undoubtedly strongly affected, if not determined, by diurnal variations in temperature, light, and the time of feeding. As already explained, in the present experiments the temperature was thermostatically maintained at 20° C, the daily light period was from about 08.00 hr. till about 18.00 hr., and the time of feeding was between 09.00 and 10.00 hr. It may also be added that sufficient food was always given so that an excess remained in the boxes at the end of each day.

The apparatus set up to record the waking and resting periods consisted of a wooden box containing two compartments connected by a small hole which was just large enough for the passage of one animal at a time. Into this hole was suspended a hinged bar which was pushed aside whenever a mouse passed. The bar was attached to a sprung arm by means of which its every movement was recorded on a smoked drum. In this way the spontaneous activity of the animals could be assessed in figures for every hour of the day and night. It was found convenient to keep five mice in the apparatus at the one time, and in order that they should be forced to visit both chambers of the box, the food supply was kept in one and the water supply in the other.

With this apparatus recordings of spontaneous activity were taken for 20 days, one group of five males occupying the box for the first 10 days and another for the second 10 days. The average figures for each of the 24 hr., representing the average numbers of times the animals passed through the hole, are given in table 20 and expressed in graph 19, figure 6. As might be expected in an experiment involving the hourly measurement of spontaneous bodily activity, the day to day variation was very high. However, although the differences between the mean figures for successive hours are never statistically significant, the larger differences, and of course those between the maxima and minima, are highly significant. The form of the graph obtained from the averages is reasonably smooth, and its broad outline must be considered as reliable. There is evidently a period of high activity associated with the feeding time between 09.00 and 11 00 hr , and an afternoon period of rest which is most marked between 13 00 and 16.00 hr. Thereafter activity rises to an evening maximum between 18.00 and 19 00 hr , and then falls steadily until about midnight The moderate activity of the succeeding 4 hr. is followed by a period of rest which is most marked between 05 00 and 07.00 hr.

Clearly these variations in bodily activity can be related to the variations already described in mitotic activity. A comparison between graphs 11 and 19 shows immediately that the one is the inverse of the other. This comparison is traced in detail, and its importance discussed, in the section below.

TABLE 20. THE SPONTANEOUS BODILY ACTIVITY OF FIVE MALE MICE EXPRESSED AS
THE AVERAGE NUMBERS OF PASSAGES THROUGH A HOLE PER HOUR (SEE ALSO
GRAPH 19, FIGURE 6)

hour ending	number of counts	range	mean ± standard error
10.00	20	20–88	52·5 ± 4·92
11.00	20	6–85	44·3 ± 5·16
12.00	20	4–61	28·9 ± 3·84
13.00	20	0–50	12·9 ± 3·80
14.00	20	0–38	8·5 ± 2·14
15.00	20	0–41	6·6 ± 2·10
16.00	20	0–35	9·2 ± 2·06
17.00	20	4–77	22·3 ± 3·96
18.00	20	6–78	31·6 ± 4·77
19.00	20	0–69	35·9 ± 4·00
20.00	20	6–81	32·0 ± 4·50
21.00	20	2–64	27·7 ± 3·60
22.00	20	6–52	24·8 ± 2·64
23.00	20	3–64	20·5 ± 3·18
24.00	20	4–42	18·8 ± 2·57
01.00	20	7–49	20·9 ± 2·75
02.00	20	3–42	19·0 ± 2·35
03.00	20	0–56	19·7 ± 3·24
04.00	20	0–63	17·6 ± 3·51
05.00	20	0–33	10·9 ± 1·83
06.00	20	0–31	7·3 ± 1·58
07.00	20	0–28	9·2 ± 1·90
08.00	20	0–42	10·6 ± 2·41
09.00	20	1–41	13·9 ± 2·33
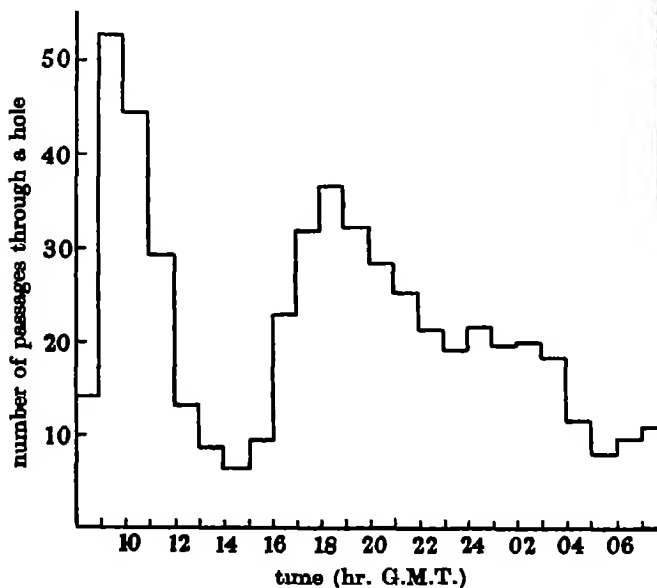
FIGURE 6. Graph 19 showing the average diurnal variations in the spontaneous
bodily activity of five male mice.

Discussion

The first conclusion which emerges from the above observations is that, in spite of a considerable amount of individual variation, there exists in many tissues of the adult male mouse a clearly defined double diurnal cycle of mitotic activity. The most accurate observations are those on the ear epidermis, pieces of which were removed periodically from living animals. These indicate that, in the conditions in which the mice were kept, there are two periods of maximum mitotic activity at 06 00 and 14.00 hr., and two periods of minimum mitotic activity at 10.00 and 20.00 hr. However, the less accurate observations on groups of mice killed at 2 hr. intervals are equally important because of their general confirmation of this result, and because they establish the further important point that the double cycle seen in the ear is common to many other tissues as well With little or no variation in timing, this cycle was observed in the epidermis of the back, the stratified epithelium of the oesophagus, the epithelium lining the tubules of the epididymis, and the proliferating zone of the duodenal mucosa Exceptions were sought for and found in the proliferating centres of the intestinal lymph nodules and in the seminiferous tubules of the testis, in both of which the extremely high rate of cell division continues without interruption day and night.

These various tissues examined fall into a natural series from the epididymis with the lowest rate of cell division to the testis with the highest. In tissues with relatively low mitotic activity there is the sharply defined double cycle in which the minimal mitosis counts approximate to zero. In more active tissues like the duodenal mucosa the double cycle is still discernible, but the minima are not so clearly marked since they are not so low. In highly active tissues like the lymph node centres and the seminiferous tubules the minima are eliminated altogether, and the graphs obtained are almost flat.

The observation that the diurnal rhythms are approximately the same in different tissues is in contradiction to the conclusion of Blumenfeld (1942) who studied the epidermis, renal cortex, and salivary gland of the rat. His results indicated that the diurnal rhythms of these three tissues are quite unrelated, and he reached the conclusion that the factors determining mitosis cycles are not common to the body as a whole. Instead, they are peculiar to each organ, or, as it should more accurately be stated, to each tissue. In the present work an attempt was made to repeat these experiments in the mouse, but conclusive results were not obtained because the rate of cell division in both the renal cortex and the salivary gland proved to be too low for accurate counting.

The second main conclusion which can be drawn from the present observations concerns the relation between mitotic activity and bodily activity. It is obvious that a remarkably close relation exists between the forms of the graphs for mitotic activity, particularly the more accurate graph 11, and of the graph obtained from the experiments on bodily activity. This relation, which is an inverse one, is brought out in graph 20, figure 7 in which graphs 11 and 19 are superimposed, and in which

the liberty has also been taken of indicating bodily activity by a line instead of, more correctly, by blocks. At 10.00 hr., when mitotic activity is low, bodily activity, associated with the feeding period, is extremely high. Mitotic activity then rises to a maximum as bodily activity falls to a minimum in the early afternoon, and falls as bodily activity rises again in the evening The minimum mitotic activity at 20.00 hr. is preceded by the maximum bodily activity just before 19.00 hr. Thereafter mitotic activity rises to a moderate figure at which it remains for some 6 hr and coincidentally bodily activity falls to a moderate figure at which it also remains for some 6 hr. In the early morning mitotic activity rises
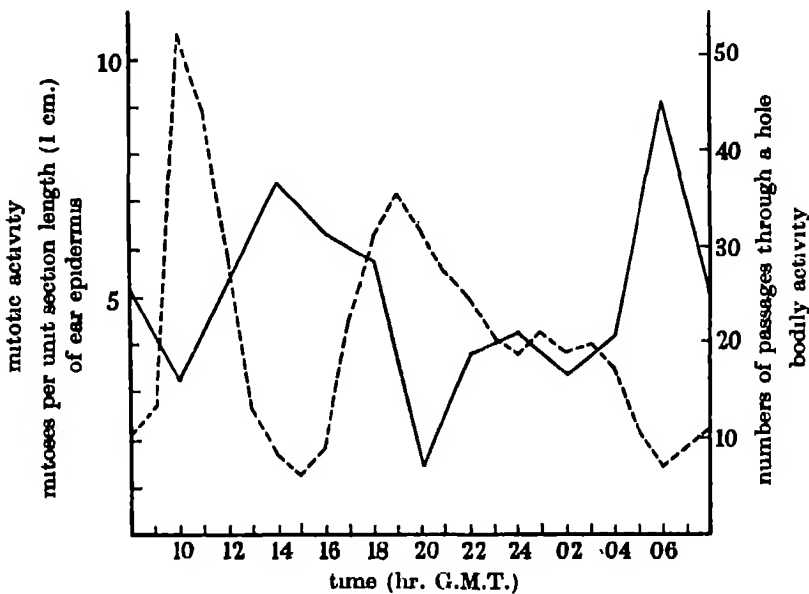
FIGURE 7. Graph 20 showing the inverse relationship between the mitotic activity of the ear epidermis (continuous line) and the spontaneous bodily activity (broken line)

sharply as bodily activity falls to a minimum between 05.00 and 06 00 hr., and then falls sharply again as the animals become active towards their feeding time. It is an inescapable conclusion that periods of high mitotic activity coincide with periods of rest, that periods of low mitotic activity coincide with periods of wakefulness and exercise, and that periods of moderate mitotic activity coincide with periods of moderate bodily activity.

In this connexion it is interesting that Riley (1937) has demonstrated in the house sparrow, *Passer domesticus* L., that spermatogenesis is most active between 02.00 and 04 00 hr when the bird is asleep, and is entirely eliminated between 07.00 and 10.00 hr. when it is awake. An artificial reversal of day and night results in a reversal of this cycle. It would appear that in this species, at least at the time when the experiments were performed, spermatogenesis does not take place sufficiently rapidly to be free of the effects of diurnal variations in bodily activity.

On the other hand, freedom from such effects in highly active tissues has been reported by Blumenfeld (1943) in epidermal carcinomata induced by methyl-cholanthrene, and freedom from the effects of the oestrous cycle has been proved by Bullough (1946) in the case of implanted mammary carcinomata.

Of course, the general conclusion that diurnal rhythms in mitotic activity are related to diurnal rhythms in bodily activity is reached only by comparing the averages of large numbers of figures, and, as already stressed, there is submerged in these averages a considerable degree of individual variation. This variation is most clearly illustrated in the results obtained from individual mice by means of earclips (graphs 1 to 10). However, it is also evident in all the other experiments from the high values of the standard errors, and here again it would seem that mitotic activity and bodily activity are linked It is common to find a box in which one or two mice are active while the others are asleep, just as it is common to find odd mice asleep while the majority of their fellows are awake. Similarly, the same group of mice may be awake at a certain hour of one day and asleep at the same hour of the next. Thus, if the incidence of mitosis is closely related to periods of sleep and of wakefulness, a high degree of variation between individual mice at any one time, and between groups of mice from day to day, is not to be wondered at but is rather to be expected

These individual and day to day variations furnish some explanation for the confusion which has surrounded the subject of diurnal mitosis rhythms in mice. Both Ortiz-Picon (1933) and Carleton (1934) described such cycles in the epidermis, but while the former indicated that the maximum occurred during the day, the latter considered that it occurred during the night. Since then Cooper & Franklin (1940) have reported that the greatest epidermal mitotic activity in their mice was at about 10.00 hr., and Blumenfeld (1942, 1943) found the maximum at 12.00 hr. in mice and at 09.00 hr. in rats. None of these papers mention a double cycle, and none of them agree with the results recorded here. However, this does not appear to be so surprising when it is remembered that there are at least three important variables involved. The first of these is the question of individual variation already discussed. The second is the question of the age, the condition, and perhaps also the sex of the animals. It cannot be expected, for instance, that mice of less than one week old, such as were used by Carleton, will show the same type of cycle as do adults. The third is the question of laboratory routine. The animals will undoubtedly be active, and the mitosis rate presumably low, at what ever time they are accustomed to being fed, and factors such as temperature and day length may also be highly important.

However, the precise form of the diurnal cycle of mitotic activity in any mouse, or in any colony of mice, is unimportant compared with a conclusion that the form of this cycle is not fixed, but is determined by the times and lengths of the periods of rest and of exercise. The final proof of this conclusion has not yet been established, but it may be obtained from the experiments on artificially induced rest and exercise which are now being carried out.

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The effects of experimentally induced rest and exercise on the epidermal mitotic activity of the adult male mouse, *Mus musculus* L.

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As a preliminary study of the conditions which affect the hour to hour variations in epidermal mitotic activity in the adult male mouse, experiments were carried out involving artificially induced sleep and exercise

In the first experiment the animals were injected with a dose of barbiturates sufficient to induce sleep for a period of 3 or 4 hr. at a time of day when they were usually active. The injections were given at 17 00 hr. as the animals were waking from their afternoon sleep, and they resulted in a sharp rise in mitotic activity A maximum was reached at 20 00 hr, the time when the mitotic activity of the controls had dropped to a minimum. As the animals recovered and became active, their mitosis rate fell quickly to a low level

In the second experiment the animals were placed in a slowly revolving box, and so forced to remain awake and active throughout an afternoon when they would otherwise have been asleep. This treatment resulted in extremely low mitotic activity, in contrast to which that of the normally sleeping controls rose to a high level. It is probably significant that when the mice were released from the revolving box, and could at last sleep, their mitosis rate remained low. This suggests that the excessive exercise had either resulted in the production of some mitosis-depressing substance which remained in the system, or that some substance vital to mitosis had been used up and took some time to be replaced.

The conclusion is now justified that the rate of epidermal mitosis normally increases during sleep, and decreases during hours of wakefulness and exercise In this way the form of the diurnal mitosis cycle is determined by the habits of the animals

INTRODUCTION

In a description of the diurnal cycle of mitotic activity in the adult male mouse (Bullough 1948), it was shown that, in spite of a high degree of individual variation, a maximum rate of cell division was usually developed at 06.00 and 14.00 hr., and a minimum at 10.00 and 20.00 hr. This was also true in a variety of internal tissues. It was further shown that the diurnal variations in mitosis are apparently related to diurnal variations in bodily activity, since, on the average, the mitosis rate was

highest at those times when the mice were usually resting and lowest at those times when they were usually active. The high degree of individual variation in mitotic activity may be related to the high degree of individual variation in spontaneous bodily activity.

Experimental investigation of these last points would appear to be of considerable interest. The object of the present paper, therefore, is to attempt to prove the connexion between rest and high mitotic activity, and between exercise and low mitotic activity

MATERIAL AND METHODS

(1) *The mice*

Most of the mice used for these experiments were of the Kreyberg white label strain, but one or two mice of Strong's *CBA* strain were also introduced into each experimental group. No differences of response were found between them. All were males of between 3 and 4 months of age, and so were in full-breeding condition. All were apparently in perfect health. Before and during the experiments they were reared and kept at a constant temperature of 20° C Their diet consisted of whole oats, dog biscuit, and oat cake soaked in cod-liver oil, and it was given to them between 09.00 and 10.00 hr. The experiments were performed in winter time when the daily light period was from 08 00 to 18 00 hr All references to the hour of day are in Greenwich mean time.

(2) *Experimental methods*

The details of the various experimental conditions to which the mice were subjected are given below in the course of the observations

(3) *Histological methods*

The methods used in removing pieces of the ear epidermis, and in fixing, sectioning and staining them, were those already described in detail by Bullough (1948).

(4) *Statistical methods*

In all cases the method of counting the numbers of epidermal cell divisions was the same. To avoid the danger of including the same division twice, the mitoses were counted in alternate serial sections. All stages of mitosis from prophase to telophase were included in the counts which were made on lengths of 1 mm. sections cut 7μ thick As the counts for each group of 10 mm. were completed, they were added together to give the figure for one unit section length of 1 cm. From each earclip ten separate counts were made, each for a section length of 1 cm., and therefore from each group of five earclips fifty counts were taken. From these fifty figures the averages and standard errors were obtained by the method already described (Bullough 1948).

In the supplementary observations on the hour to hour changes in bodily activity, records were taken throughout six repetitions of each experiment, different

mice being used each time. The activity for each hour was then taken to be the average of the six figures for that hour. The standard error was calculated from the standard deviation σ which was obtained by the use of the formula $\sigma = \sqrt{(\Sigma f d^2/N - 1)}$, where f is the frequency, d the deviation from the mean, and N the number in the sample. This is the formula recommended for small samples by Simpson & Roe (1939).

<center>OBSERVATIONS</center>

<center>(1) *Effect of rest*</center>

In this first experiment the effect was determined of an artificially induced rest at a time when the mice would normally be very active. Five male mice were put to sleep by means of a subcutaneous injection of 'somnifaine' (a solution of the diethylamine salts of diethyl- and allyl-isopropyl-barbituric acids). Preliminary experiments were carried out to determine what dose was necessary at 17.00 hr., when the mice were waking from their afternoon sleep, to induce further sleep for a period of about 3 hr. The results indicated that 2·5 mg. was the optimum dose. A lower dose of 2·0 mg did not ensure that all the animals would succumb fully, and a higher dose of 3·0 mg caused too deep a sleep for too long a period As it was, one animal responded too deeply to the dose of 2·5 mg and had to be replaced. In this animal mitotic activity fell to a low level, and it is omitted from the results tabled below.

The experiment was commenced at 16.00 hr. with the removal of the first earclips, and at this time the animals were already becoming active after their usual afternoon sleep. At 17.00 hr the injections were given. After 15 min the animals were staggering about the box, and after 30 min. they were fast asleep. At 18.00 and 20.00 hr. the second and third earclips were taken, and all this time the mice remained asleep, their only movements being deep breathing and occasional scratching, twitching, or wriggling. At various times between 20.00 and 22.00 hr. they began to recover, and when the fourth earclips were taken at 22.00 hr., all the animals were tottering about in an uncertain manner By 24.00 hr., when the fifth earclips were taken, they were almost fully recovered and were very active. The same was true at 02.00 hr. when the sixth and last earclips were taken

An analysis of the mitotic activity shown by these earclips is given in table 1. Also included in the table are the figures for normally active mice at the times covered by the experiments.

These two sets of figures are illustrated and compared in figure 1. In the controls the mitosis rate dropped as the animals awoke and became active in the late afternoon, while in the drugged and sleeping mice the rate rose. The differences between the two sets of figures are not statistically significant by 18.00 hr., but by 20.00 hr. the numbers of cell divisions in the control mice had reached a minimum while those of the drugged mice had reached a maximum. Then the difference between the figures is strongly significant, and it is obvious that the artificial induction of sleep resulted in a great increase in the mitosis rate at a time when that rate would

normally be reduced. The maximum rate of cell division, developed after 3 hr. of drugged sleep, was not quite so high as that reached in normal sleep (Bullough 1948), although it is possible that had the effects of the drug continued for another hour or two, a higher rate might have been reached. As stated above, the mice recovered consciousness before 22.00 hr., and thereafter they continued to be active. The recovery was accompanied by a sharp drop in mitotic activity.

TABLE 1. AVERAGE NUMBERS OF MITOSES PRESENT PER UNIT LENGTH (1 CM.) OF SECTIONS OF THE EAR EPIDERMIS OF TEN CONTROL MICE AND OF FIVE MICE INJECTED AT 17.00 HR. WITH BARBITURATES

time of day (G.M.T.)	control mice			drugged mice		
	number of counts	range	mean ± standard error	number of counts	range	mean ± standard error
16.00	100	1–14	6·4 ± 0·09	50	1–9	4·0 ± 0·29
18.00	100	0–16	5·9 ± 0 10	50	2–9	4·6 ± 0·24
20.00	100	0–7	1·6 ± 0 04	50	3–11	6·8 ± 0·31
22.00	100	0–10	3·8 ± 0·10	50	0–4	2·4 ± 0·14
24.00	100	0–11	4 3 ± 0 07	50	0–4	2·6 ± 0·13
02.00	100	0–11	3·4 ± 0 07	50	0–5	2·3 ± 0·20
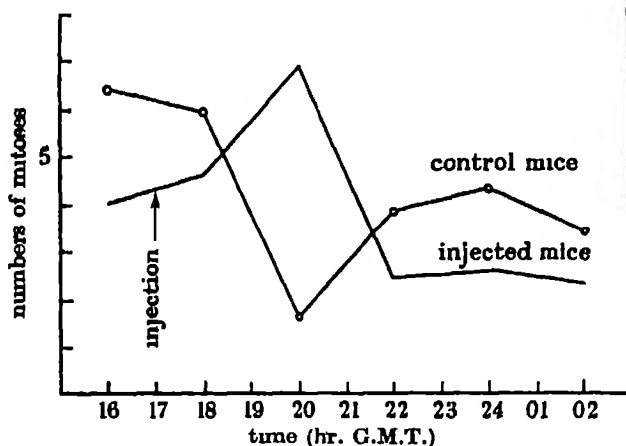
FIGURE 1. Graph 1 showing the average numbers of mitoses per cm. length of sections of ear epidermis in control mice and in mice which at 17.00 hr. were put to sleep by means of barbiturates.

To emphasize these results still further, groups of five male mice were drugged in the same way at the same time of day, and their times of waking and degrees of bodily activity were measured. This was done, as already described by Bullough (1948), by putting them into a box divided into two compartments. These two compartments communicated with each other by a small hole through which only one animal could pass at a time. In the hole was suspended a lever linked to a sprung arm by which every movement was recorded on a revolving smoked drum. The number of movements per hour, representing the number of times the mice

passed through the hole, was then used as a measure of bodily activity. The average results of six separate experiments performed with this activity box are recorded in table 2, in which are also included analyses of the movements of normal mice at these times.

TABLE 2. THE SPONTANEOUS BODILY ACTIVITY OF FIVE CONTROL MICE AND OF FIVE MICE INJECTED WITH BARBITURATES AT 17.00 HR. EXPRESSED AS THE AVERAGE NUMBERS OF PASSAGES THROUGH A HOLE PER HOUR

hour ending (G.M.T.)	control mice			drugged mice		
	number of counts	range	mean ± standard error	number of counts	range	mean ± standard error
16.00	20	0–35	9·2 ± 2 06	6	2–15	7 3 ± 1·91
17.00	20	4–77	22·3 ± 3 96	6	5–30	16 0 ± 3·82
17.30	—	—	—	6	10–39	26 9 ± 4 33
18 00	20	6–78	31 6 ± 4·77	6	—	0 - -
19.00	20	0 69	35·9 ± 4·00	6	—	0 —
20.00	20	6–81	32 0 ± 4·50	6	—	0 —
21.00	20	2–64	27 7 ± 3·60	6	0–9	3 5 ± 1·54
22.00	20	6–52	24 8 ± 2 64	6	0–26	11·2 ± 3·82
23.00	20	3–64	20 5 ± 3·18	6	5–31	20·2 ± 2·96
24.00	20	4–42	18·8 ± 2 57	6	2 43	17 2 ± 6 17
01·00	20	7–49	20·9 ± 2·75	6	7–41	18·3 ± 5·21
02.00	20	3–42	19 0 ± 2·35	6	13–37	25 0 ± 4·16

In graphs 2 and 3, figure 2, comparisons are made between the mitotic activity and the bodily activity of the control mice and of the drugged mice. For clarity, the liberty is taken of representing the results for bodily activity as line graphs. Because of this, the comparison made is really between the mitotic activity at any one time and the total bodily activity occurring in the previous hour. The close but inverse relationship between cell division and exercise is strikingly illustrated. By 18.00 hr., after only 30 or 40 min. sleep, the mitosis rate of the drugged mice already appeared to be rising, but the maximum, noted at 20 00 hr., evidently took some 2 hr. to develop. The drop in the mitosis rate following the resumption of exercise was more rapid, so that a minimum was reached by 22.00 hr. after about 60 min. wakefulness. Thereafter, as bodily activity continued high, mitotic activity remained low.

The general conclusion would seem to be that when mice are put into a light sleep by means of barbiturates the mitosis rate rises as it does in normal sleep. However, the necessary dosage of barbiturates must be carefully determined. If, as was shown in discarded preliminary experiments, the dose given is too high, the result is a fall in the numbers of cell divisions.

(2) *Effect of exercise*

The attempt to induce the converse effect to that recorded above was made with the use of a revolving box. By means of this a treadmill effect was obtained, and the mice were forced to keep awake and active at a time when they would otherwise

have been asleep. The box was wooden with a perforated zinc top held in place by
four catches, and it was made to rotate round its long axis by a drive from a clock-
work motor. It made one complete revolution every 5 min., which was sufficient to
prevent sleep but which did not cause excessive fatigue.
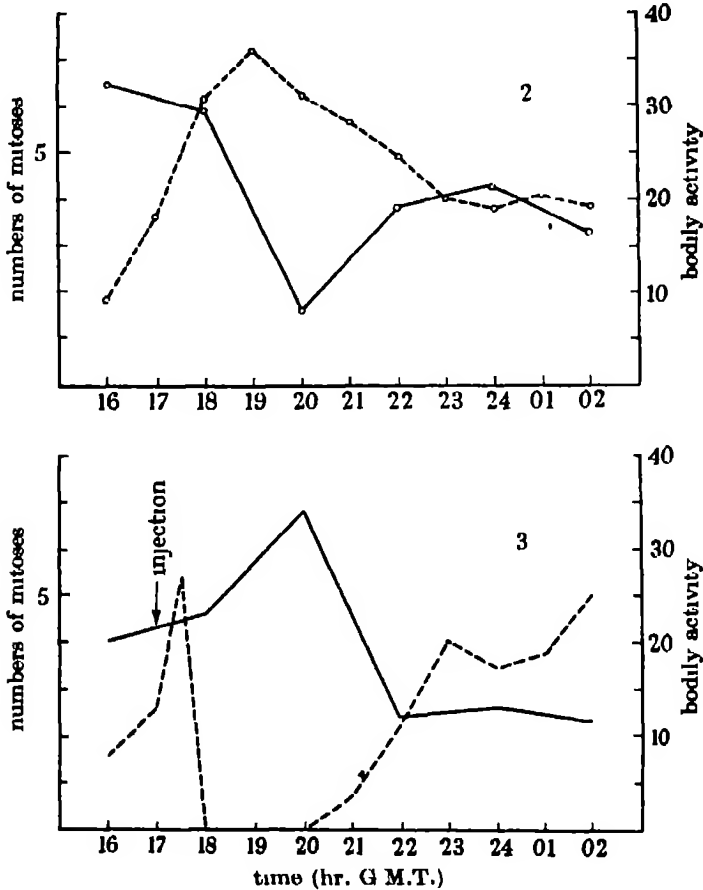
FIGURE 2   Graph 2 showing the inverse relationship between epidermal mitotic activity
(continuous line) and bodily activity (broken line) in control mice   Graph 3 showing the
inverse relationship between epidermal mitotic activity (continuous line) and bodily activity
(broken line) in mice which were put to sleep by means of an injection of barbiturates at
17 00 hr.

Five adult males were placed in this box at 11.00 hr., which is a time when,
feeding being over, they would normally be settling down in readiness for their
early afternoon sleep. The mice chosen had already begun to rest in a corner when
they were transferred to the revolving box. The very act of placing them into a new
box was sufficient to stimulate them greatly so that they immediately became
extremely active. This activity continued almost undiminished until 15.00 hr , when
the first signs of tiredness were apparent. From 15.30 until 17.00 hr , when the box

was stopped, the mice adopted crouching attitudes and walked as slowly as possible while keeping pace with the revolutions. At 17.00 hr. the mice were returned to their old box when, instead of immediately falling asleep as was anticipated, they spent about 30 min. feeding eagerly. Hunger was obviously their dominant feeling at this time in spite of the fact that a little food had been available to them in the revolving box Then, after their hunger had been relieved, they even found some energy to fight However, by 18.00 hr they had all fallen asleep, and they remained so almost until the end of the experiment at 20.00 hr

TABLE 3. AVERAGE NUMBERS OF MITOSES PRESENT PER UNIT LENGTH (1 CM.) OF SECTIONS OF THE EAR EPIDERMIS OF TEN CONTROL MICE AND OF FIVE MICE KEPT IN A REVOLVING BOX FROM 11 00 UNTIL 17 00 HR

time of day (G.M T)	control mice			exercising mice		
	number of counts	range	mean ± standard error	number of counts	range	mean ± standard error
10 00	100	0–11	3 2 ± 0 24	50	0 6	2 5 ± 0 23
12 00	100	0 14	5 4 ± 0 10	50	0–2	0 4 ± 0 01
14 00	100	1–18	7 4 ± 0 09	50	0–2	0 4 ± 0 01
16 00	100	1 14	6 4 ± 0 09	50	0–2	0 6 ± 0 01
18 00	100	0–16	5 9 ± 0 10	50	0–4	0 9 ± 0 15
20 00	100	0–7	1 6 ± 0 04	50	0–3	1 0 ± 0 10
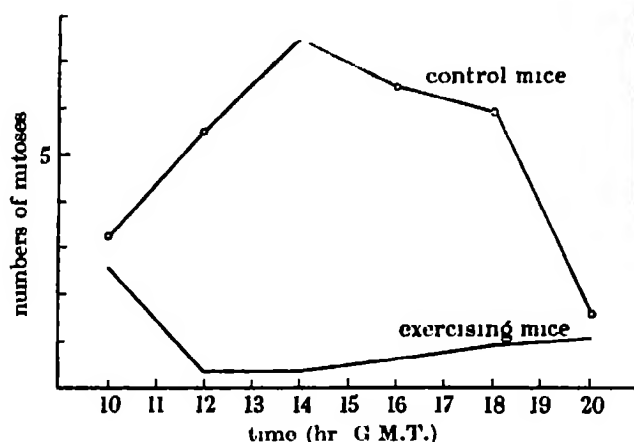
FIGURE 3. Graph 4 showing the average numbers of mitoses per cm length of sections of ear epidermis in control mice and in mice given exercise in a revolving box from 11.00 until 17.00 hr.

The removal of earclips from these mice started at 10 00 hr. and continued at 2-hourly intervals until 20.00 hr. In table 3 there are recorded the average numbers of mitoses found in these clips, as well as the numbers of mitoses present in earclips of control mice not subjected to forced exercise.

The comparison between these two sets of figures is made in graph 4, figure 3, and it is evident that, with the elimination of the usual afternoon sleep period, the

mitotic activity of the ear epidermis remained low. Indeed, it is notable that after only 1 hr. in the revolving box the mitosis rate fell sharply to a figure which is a long way below any previously observed minimum. Indeed, the treatment depressed mitosis almost to the point of elimination. It should also be noted that the depression achieved was not overcome by the effects of the 2 or 2½ hr. sleep which preceded 20.00 hr., and the impression is therefore gained that some extreme exhaustion was produced which would take time to be overcome.

Finally, the experiment was repeated six times with different groups of five mice each, and the spontaneous bodily activity of the animals was measured in the hours before and after the period spent in the revolving box. The measurement was made by means of the same activity box mentioned above. The results are given in table 4 which, for comparison, also includes the average figures for the spontaneous bodily activity of normal mice at the same times of day.

TABLE 4. THE SPONTANEOUS BODILY ACTIVITY OF FIVE CONTROL MICE AND OF FIVE MICE KEPT FROM 11.00 TO 17.00 HR. IN A REVOLVING BOX EXPRESSED AS THE AVERAGE NUMBERS OF PASSAGES THROUGH A HOLE PER HOUR

hour ending (G.M.T.)	control mice			exercising mice		
	number of counts	range	mean ± standard error	number of counts	range	mean ± standard error
10.00	20	20–88	52·5 ± 4·92	6	24–57	43·2 ± 5·15
11.00	20	6–85	44·3 ± 5·16	6	23–48	40·3 ± 3·87
12.00	20	4–61	28 9 ± 3 84			
13.00	20	0–50	12·9 ± 3·80			
14.00	20	0–38	8·5 ± 2·14		in revolving box	
15.00	20	0–41	6 6 ± 2·10			
16.00	20	0–35	9 2 ± 2 06			
17.00	20	4–77	22·3 ± 3 96			
17.30	—	—	—	6	15–41	29·3 ± 3 83
18 00	20	6–78	31·6 ± 4 77	6	0–3	0·5 ± 0·51
19.00	20	0–69	35·9 ± 4·00	6	—	0 —
20.00	20	6–81	32 0 ± 4·50	6	0–12	4·7 ± 1·92

In graphs 5 and 6, figure 4, the comparisons are made between the mitotic activity and bodily activity of the control and of the experimental mice. Again, the way in which high bodily activity is associated with low mitotic activity is emphasized, and again it is evident that the rest period of the experimental animals from about 17.30 hr. until about 20.00 hr. was not sufficient to cause any significant rise in mitotic activity.

DISCUSSION

These results supplement and strengthen the conclusions reached by Bullough (1948) in his recent study of the normal diurnal cycle of mitotic activity in the adult male mouse, and constitute a proof of the inverse relationship between mitotic activity and bodily activity. Although in the present work only the epidermis was

examined, it may be presumed that the effect is a common one, since it has already been shown that many mouse tissues follow diurnal cycles of mitotic activity identical with that of the epidermis.
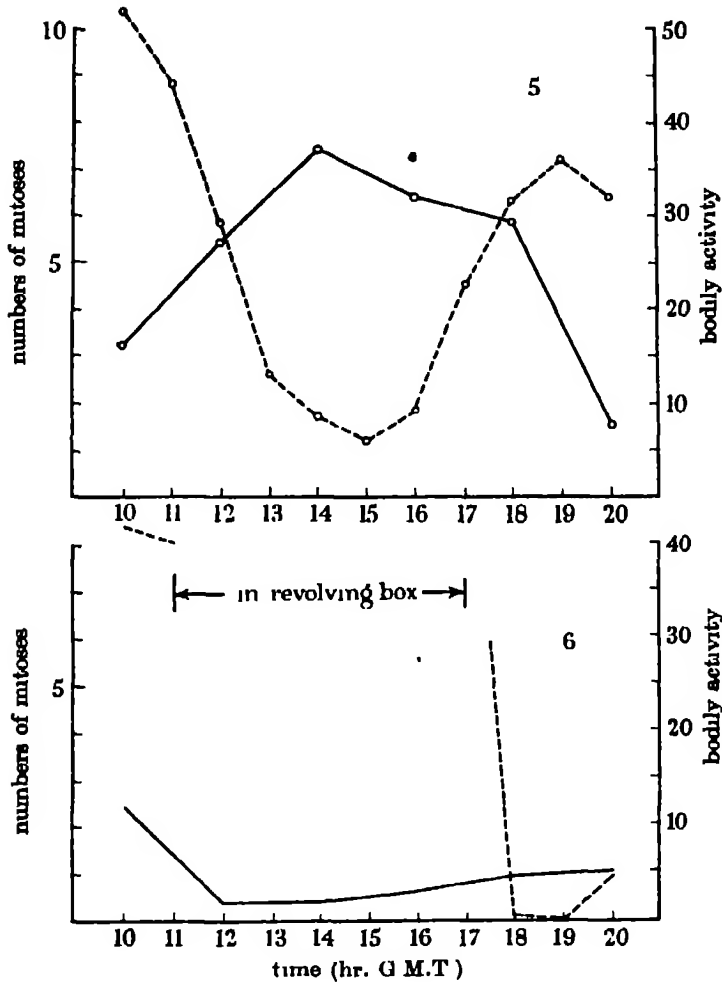
FIGURE 4. Graph 5 showing the inverse relationship between epidermal mitotic activity (continuous line) and bodily activity (broken line) in control mice. Graph 6 showing the inverse relationship between epidermal mitotic activity (continuous line) and bodily activity (broken line) in mice given exercise in a revolving box from 11.00 until 17.00 hr.

From this conclusion that high mitotic activity is associated with sleep and low mitotic activity with exercise, it follows that no general statement can be made on the normal form of the diurnal mitosis cycle of laboratory mice. The type of cycle which is common in any one colony must be entirely determined by the habits of resting and waking of its members, and these in turn will be determined by the routine of the laboratory. It is very probable that the form of cycle will also prove

to be strongly affected by such factors as the age, the sex, and the diet, or, in other words, by the general condition of the mice.

The way is now open for an examination of the effects on mitosis of some of the factors which are known to vary between sleeping and waking. The first such factor which comes to mind is the physical one of body temperature which is, of course, closely associated with the more complicated subject of metabolic rate. Both body temperature and metabolic rate are known to be higher in waking than in sleeping, and a lower body temperature, or a slower metabolic rate, may therefore be important prerequisites for active mitosis. In this connexion it will be remembered that cell division in the mammalian testis cannot take place actively unless that organ comes to lie in the cooler conditions of the scrotal sac, an observation which may be compared with that of Riley (1937) that spermatogenesis in the house sparrow, *Passer domesticus* L., only proceeds at a high rate during the night when body temperature is lowered. However, it does not appear that body temperature can be the only important factor in diurnal mitosis rhythms. The outcome of the revolving box experiment seems to indicate that excessive exercise, or heightened metabolic rate, results either in the production of a mitosis-depressing substance which takes some hours to be eliminated, or in the using up of some mitosis-stimulating substance which takes some hours to reform in sufficient quantity. In other words, it may well be that the most important factor, or factors, are chemical rather than physical, and work on this point is now in progress.

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The quantitative investigation of the vertebrate brain and the applicability of allometric formulae to its study

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A new method for the estimation of the parameters in the allometric equation is described whereby both the estimates and their standard errors are determined in a simple manner The fitting of curves to biological data is discussed, and the lack of equivalence between a form containing exponential parameters and its logarithmic transformation shown The classical method is extended to apply to this situation

The Dubois-Lapicque-Brummelkamp theory is discussed in the light of this work and shown to be untenable   In particular, there is no basis for the view that, if the form $y = bx^\alpha$ is fitted to the brain and body weights of vertebrates, the interspecific value of x is ⅔ and its intraspecific value is 0·26 A final maximum brain weight is demonstrated for some species which is independent of the adult body weight

The analysis of mental development in terms of 'cephalization constants' is not in accordance with the facts, and a much deeper analysis of the comparative anatomy involved must be made if a quantitative study of the ontogenetic and phylogenetic development of the brain is to be achieved

INTRODUCTION

Biologists have become increasingly aware that, while the qualitative description of phenomena is a necessary step in anatomy and physiology, the attempt must be made to discuss these sciences quantitatively and, moreover, to use a mathematical approach to their problems We are no longer satisfied with saying that the bodily proportions of a newly-born primate differ from those of the adult in certain respects, but we attempt to make precise quantitative statements about the changes and strive to account for them (Medawar 1944) Whenever an experiment is performed, the results are plotted and a search is made to find precise relationships between the variables. Unfortunately, unless great care is taken, the result may be of little value

If we state the problem a little differently, we may perhaps say that we try to find a formula which will not only describe the relationships found in the experiments but will predict possible relationships which are as yet unknown or will at least lead to the design of useful experiments There are two ways of procedure. We may formulate a hypothesis which can be expressed in mathematical terms, probably as a differential equation, and then, perhaps, investigate the equation and find 'a formula'. An experiment is designed and the formula fitted to the results Statistical methods enable us to test whether the results of the experiment differ significantly from those expected by the hypothesis. If the results do so differ, the hypothesis must be rejected; on the other hand, if the experiment does not cause us to reject the hypothesis, we may continue to examine its implications Alternatively, we may merely take our experimental results and fit some empirical formula to them. In this case it must be remembered that there is no unique formula

(the 'true' formula) which can be fitted to a set of points—we may fit polynomials, trigonometric series, functions of exponentials, etc., and the best we can hope for is that one of these formulae may suggest a theoretical approach which may be tested in the manner first described. It will be remembered that it is quite easy to calculate a polynomial the graph of which will pass exactly through all the points plotted from an experiment; but it is unlikely that such a polynomial will serve any useful function as a description of the mechanism underlying our experimental results, since we shall have to find a biological interpretation inductively for each parameter which occurs.

It is obvious that since all experimental results are subject to error, statistical methods must be used for fitting the appropriate formulae, and these methods will enable adequate estimates of the parameters and of their standard errors to be made. Unless such a process is used, the resulting formulae will be useless; if, on theoretical grounds, we make a statement about the value of some parameter in the formulae, no amount of experimentation or plotting will enable us to test the truth of this hypothesis unless correct statistical methods are used.

Again, the assumption is often made that if we have *a priori* reasons for considering several hypotheses, each symbolized by a formula, then the 'true' formula will be that which fits the data best. The falsity of such an assumption has been discussed many times, and Feller (1939) illustrates the matter very clearly by examining Gause's experiments on species of *Paramecium*. These experiments were designed to verify the well-known Volterra equations for the growth of populations, and Gause considers that his work provides this verification. Feller easily finds a 'random' formula which will fit the data more closely than the Volterra equation, and he points out that the experiments no more prove the adequacy of Volterra's theory than that of his random function.

THE RELATIONSHIP BETWEEN BRAIN WEIGHT AND BODY WEIGHT

For many centuries the organization and function of the brain have provided fascinating problems for investigation, and the problem of finding some simple relationship between the brain weight of animals and the positions of those animals on the phylogenetic scale has always been attractive; many attempts have been made to find a solution which would commend itself to scientists. One theory has met with wide acceptance and has its origin in the work of Snell (1891). This investigator suggested that if an animal weighed x units and its brain y units, then the relationship between x and y could be expressed in the form

$$y = bx^\alpha, \tag{1}$$

where b and α are real numbers.

Relationships of this form have become very familiar through the work of Huxley (1932), and its application to certain types of data has been fully discussed by Reeve (1940). This author was fully aware of the assumptions involved and his work does not enter into our discussion. The present paper is concerned with the manner

in which the relationship (1) has been used in connexion with certain data. The problem was also further discussed by Richards & Kavanagh (1945) and Reeve & Huxley (1945). We shall not be concerned with the general theory but only with a specific application of it which has been widely accepted.

Towards the end of the last century Dubois (1897) and Lapicque (1898) simultaneously developed a theory based on the values of α and b in (1). A large number of papers was produced by these and other authors (Dubois 1914; Lapicque 1907; Lapicque & Girard 1905, Hrdlička 1905) culminating in those of Brummelkamp (1939), while the problem is also discussed by Sutter (1943). Very adequate summaries of this work have been given by Kappers (1929), de Beer (1940) and a concise account is to be found in Huxley's book (1945) on evolution.

But the most recent exposition seems to be that of Count (1947), whose paper contains an extensive bibliography. This author examines these theories at some length but from a somewhat different point of view from that adopted here; he also produces a new formula which will be discussed later.

The theory proposed by Dubois and his successors may be briefly summarized under three headings.

(1) The parameter α is constant for groups of adult animals of different species within a genus or family and has the value $\frac{5}{9}$; this parameter is known as the phylogenetic constant.

(2) For adult animals of the same species but of different sizes, the value of α is constant and equal to 0·26 and for such intraspecific groups is known as the ontogenetic constant.

(3) The parameter b is known as the cephalization coefficient, and according to Brummelkamp b is an integral power of $\sqrt{2}$.

Dubois transformed (1) into the form

$$\log y = \log b + \alpha \log x, \tag{2}$$

and writing
$$\alpha_{ij} = \frac{\log y_i - \log y_j}{\log x_i - \log x_j} \quad (i, j = 1, 2, .. , n; \; i > j)$$
$$= \alpha_q$$

he then selected 'suitable' pairs of values, say k $(k < n)$, calculated for each pair the α_q $(q = 1, 2, ..., k)$ and finally put

$$\alpha = \frac{1}{k} \sum_{q=1}^{k} \alpha_q.$$

He found that the value of α was approximately 0·56, but he did notice that for his own measurements on bats the value of α was much higher. This method was ultimately used for many groups of animals, and it was concluded that the 'true' value of α was $\frac{5}{9} = 0·56$. This value was then treated as a kind of universal constant and used for calculating b for any given pair of values of y and x. Various hypotheses as to the magnitude and relationships of the cephalization coefficients were then elaborated, and the theory seems to have reached its climax with Brummelkamp's developments.

Here, for example, for ungulates, the logarithms of a number of brain and body weights are plotted and a set of parallel straight lines of slope ⅔ are drawn, cutting the y axis at points $\frac{1}{\sqrt{2}}$, 1, $\sqrt{2}$, 2, . . ., and since a number of the plotted points lies near one or other of these lines, it is considered that it is legitimate to conclude that the logarithms of the cephalization coefficients differ by integral multiples of $\sqrt{2}$. It must be noted that in Brummelkamp's treatment the points falling about one of these lines are treated as forming a 'natural' group; such grouping of animals does not give rise to a classification comparable with that accepted by most taxonomists.

Leaving aside these speculations of Brummelkamp, let us consider the earlier work. Even if it be granted that there are adequate reasons for justifying the fitting of a relationship of the form (1) to the data, it is obvious that two major criticisms may be levelled against the methods which have been used. In the first place, the arbitrary choice of 'suitable' values or 'suitable' groups for the determination of the parameters must vitiate any general theory and, as von Bonin (1937) has pointed out, begs the whole question of the cephalization coefficient being some kind of measure of mental development. Secondly, even if we accept the restriction on the data to be used, the method used for estimating the values of the parameters from that data is utterly faulty. It is true that modern statistical methods were not available fifty years ago but the work of Gauss, for example, would have provided a guide. Moreover, apart from the inefficient method of estimation, no kind of significance test could be derived and consequently it would be impossible to make any comparisons between different estimates of the parameters.

I have made a brief criticism of this work elsewhere (Sholl 1947) and propose to examine it here in more detail. Previously I made attempts to adapt the data to make allowance for values which were known to be the means of an unspecified number of observations; in this paper I shall first consider the actual data given by Brummelkamp-Dubois as forming the foundation of their work. It is difficult always to know why only a portion of the data for normal animals published by Hrdlička (1905) and Spitzka (1903) is used. However, it seemed advisable to use the figures these authors publish, but I have later augmented their data in order to establish more general conclusions. It will be noted that some of the 'groups' contain few animals—so few that one might hesitate to generalize from the results. It should be remembered that I am not concerned with substantiating any new theory, but, assuming that it is legitimate to fit the data by the equation (1), I shall investigate whether the interpretation which has been placed on the parameters is justified by the data.

CURVE FITTING IN GENERAL

If we propose to fit any curve to data it is essential that the process should be systematic and yield estimates of the parameters which are statistically satisfactory together with efficient estimates of their standard errors. Although the question

has been widely discussed, many investigators still fail to appreciate that it is quite impossible to give exact values for the parameters. The 'true' values of the parameters are 'population' values which have to be estimated from samples, consequently the values which are found from a sample must be subject to error—the estimate of this error is known as the standard error of the estimate. It is impossible to find the 'true' value, which, of course, would have a standard error of zero.

Before translating the method into mathematical notation we may discuss it briefly in general terms When we fit a curve to points we must first decide on the kind of curve which we consider useful for our purpose, since no method will tell us which is the 'right' curve. In fact, if we take the most obvious type of curve we should be led to some kind of polynomial, i.e. something of the form

$$y = a + bx + cx^2 + dx^3 + \ldots,$$

for if there are n observations, i e. n points on the graph, then a polynomial of degree $(n-1)$ can be found which will pass through all the points exactly. For example, given two points, we can find values for a and b so that

$$y = a + bx$$

will pass through the points. Similarly, given three points, we can find values (i.e. estimates) of a, b and c such that

$$y = a + bx + cx^2$$

will pass through all these points, and so on. As a rough generalization, we may say that apart from the linear form $y = a + bx$, polynomials are of limited value for this type of scientific inquiry. Usually, we have some reason for thinking that an equation of some particular form is applicable to our data, in other words, we have some theory as to the manner in which the observations are related. In the present case we are assuming that there are adequate grounds for considering the form $y = bx^\alpha$ to be suitable We have, then, to use a method which will enable us to estimate the best values to take for b and α. There are various ways by which this could be done, but the method of maximum likelihood has advantages which make it especially valuable. If we apply this method to our problem it leads us to the conclusion that we shall obtain the best estimates for b and α by minimizing

$$\Sigma(y - bx^\alpha)^2,$$

where the summation is taken over all the observations. In fact, this solution is often known as the least squares solution.

When we have found our estimates for b and α we could take our observed values of x and using the formula

$$y = bx^x$$

calculate for each of these x values a corresponding value, say y'. Then for each x we should have an observed y and a calculated y', and, likewise for each x, we can

find the value of $(y - y')$; this number would then be squared to ensure that we should always have positive quantities. We find the values of these squares for all the x's and add them up, i.e. sum over all the observations. It is obvious that the closer our line fits the points, the smaller will be this sum of squares. Our method is to estimate b and α so that this sum is as small as possible, i.e. by minimizing

$$\Sigma(y - y')^2 \quad \text{or} \quad \Sigma(y - bx^\alpha)^2. \tag{3}$$

This method will also enable us to find the standard errors of b and α, but it must be remembered—and this is the point which is often overlooked—we are assuming that for a given value of x, the values of y are normally distributed about a mean. In the case where we minimize

$$\Sigma(\log y - \log b - \alpha \log x)^2, \tag{4}$$

the assumption is that the logarithms of y are normally distributed, and it is not the case that if y is normally distributed $\log y$ has the same distribution. We shall see that the estimates for b and α found under these two assumptions are different.

Actually (3) leads us into difficulties of a practical nature, and while it is false to assume that (4) is an equivalent form, it is shown in the appendix that

$$\Sigma y^2(\log y - \log b - \alpha \log x)^2 \tag{5}$$

may be taken as equivalent, i.e. we may take logarithms if we use y^2 as a weighting factor. It must not be assumed that we are saying that it is 'wrong' to fit (4); we are only maintaining that the assumptions which are made in that case are quite different from those made in fitting (3) or (5). In the following section the matter is discussed somewhat more technically and the solutions for the estimates are stated.

TECHNICAL METHODS

I have used the method of maximum likelihood which, as is well known, leads to the solutions obtained by the method of least squares. In the case of the curve

$$y = bx^\alpha,$$

the method amounts to finding those values of the parameters which make (3) a minimum, the summation being made over all the observed values. Similarly, if we fit

$$\log y = \log b + \alpha \log x,$$

the parameter estimates will be found by minimizing (4). It is not the case that (3) and (4) are equivalent. Estimations by means of the form (3), where one or more parameters occur non-linearly cannot be dealt with by classical regression methods and demand the location of a root of the eliminant in the equations found by partial differentiation, and if there are more than a dozen observations the location of this root leads to calculations which are almost prohibitive. Moreover, the only estimates of the variance of the parameters which can be found are minimum variances.

The relationship $y = bx^{\alpha}$ is of considerable importance in many branches of natural science, and it seemed worth while to spend time in trying to find a method whereby not only would the very real difficulties of computation be overcome, but also a means found for calculating the standard errors of the estimates of the parameters. Such a method has been found, and its mathematical justification will be found in the appendix. The method to be adopted consists in minimizing (5) and since the function with which we are now concerned is linear in the parameters, there is no further difficulty in estimating the parameters or finding their standard errors. It must be emphasized that the results found from the minimization of (5) are quite different from those found by minimizing (4).

Alternatively, we may say that (3) assumes a normal distribution of y and we are considering the regression of y on x, while (4) assumes that $\log y$ is normally distributed and we are considering the regression of $\log y$ on $\log x$. We shall not consider which, if either, of these assumptions is justified, but we shall examine the consequences of both assumptions. The form (4) will be referred to as 'the standard logarithmic method' (s.L M.) and the form (5) as 'the weighted logarithmic method' (w L.M.).

For convenience we shall write $\log = Y$, $\log x = X$, $\log b = B$ and assume that we have n observations Summations are to be taken over all observations.

Standard logarithmic fitting

The normal equations are

$$Bn + \alpha \Sigma X = \Sigma Y, \quad B\Sigma X + \alpha \Sigma X^2 = \Sigma XY$$

With the usual notation for the variance-covariance matrix

$$c_{11} = \frac{\Sigma X^2}{n\Sigma X^2 - (\Sigma X)^2} = \left(\frac{1}{n} + \frac{\bar{X}^2}{\Sigma(X - \bar{X})^2}\right),$$

$$c_{22} = \frac{n}{n\Sigma X^2 - (\Sigma X)^2} = \frac{1}{(\Sigma X^2 - [\Sigma X]^2/n)}.$$

The residual sum of squares

$$R = \Sigma(Y - \bar{Y})^2 - \frac{[\Sigma(Y - \bar{Y})(X - \bar{X})]^2}{\Sigma(X - \bar{X})^2}.$$

Standard error of estimate of $\alpha = \sqrt{\left(\frac{R}{n-2}c_{22}\right)}.$

Standard error of estimate of $B = \sqrt{\left(\frac{R}{n-2}c_{11}\right)} = S_B.$

Standard error of estimate of $b = \dfrac{b \times S_B}{\log_{10} e} = \dfrac{b \times S_B}{0 \cdot 4343}.$

Weighted logarithmic method

The normal equations are

$$B\Sigma y^2 + \alpha\Sigma y^2 X = \Sigma y^2 Y,$$

$$B\Sigma y^2 X + \alpha\Sigma y^2 X^2 = \Sigma y^2 X Y,$$

$$c_{11} = \frac{\Sigma y^2 X^2}{\Sigma y^2 \Sigma y^2 X^2 - (\Sigma y^2 X)^2},$$

$$c_{22} = \frac{1}{\Sigma y^2 X^2 - (\Sigma y^2 X)^2/\Sigma y^2},$$

$$R = \Sigma y^2 (Y - \overline{Y})^2 - \frac{[\Sigma y^2 (Y - \overline{Y})(X - \overline{X})]^2}{\Sigma y^2 (X - \overline{X})^2}.$$

The standard errors will be found as shown above for the s L M.

Data. The tables given by Brummelkamp (1939), pp. 189, 265–266, 270) for rodents, ungulates, fish, Amphibia, the table of Dubois for birds.

Additional data for *Macaca* spp , Sciuridae, from Hrdlička (1905) and Spitzka (1903)

The data may be considered in three sets.

Set I. The groups considered by the Dubois school (rodents, ungulates, etc.).

Set II. Two groups of genera *Macaca*, Sciuridae.

Set III. Adult animals within the same species.

Six-figure logarithms were used and the figures were only rounded off in the final tables. The differences of the estimated values of the parameters from a given value were tested by dividing this difference by the estimated standard error of the parameter. It is well known that this quantity is distributed as 'Student's' t; the 5 % level was taken as significant

RESULTS

These are summarized in tables 1, 2 and 3, and by way of illustration, two sets of graphs have been drawn for the *Macaca* spp. Figure 1 shows the data plotted on a double logarithmic grid, i e the diagram is a plot of logarithms of the observations, while figure 2 shows a plot of the actual observations. Three lines are shown fitted to the points in each figure; one fitted by the w.L.M., the second by the s L.M. and the third that demanded by the Dubois-Brummelkamp theory. The latter calls for little comment, and which of the other two is to be preferred depends merely on the hypothesis favoured by the investigator Reeve (1940) working on the snouts of ant-eaters chose the s.L M. and gave clear reasons for this choice. It should be remembered that if there were a greater range in the values of the brain weights these lines would be even more dissimilar. If, for example, the lines for the ungulates were drawn, the line fitted by the w.L.M. would have a slope of 0·9, while that found by the s.L.M. would have a slope of 0·5.

Table 1. Estimates of α for groups

set	group	number in group	estimate of α	weighted logarithmic method					standard logarithmic method					
				standard error of estimate	$\lvert\frac{5}{6}-\alpha\rvert$	t	f	significance of t (5% level)	estimate of α	standard error of estimate	$\lvert\frac{5}{6}-\alpha\rvert$	t	f	significance of t (5% level)
I	rodents	32	0·623	0·038	0·068	2·44	30	S.	0·692	0·044	0·137	3·12	30	S.
	ungulates	56	0·912	0·041	0·357	8·73	54	S.	0·506	0·056	0·049	0·891	54	N.S.
	birds	25	0·369	0·027	0·186	6·88	23	S.	0·549	0·041	0·006	0·156	23	N.S.
	Amphibia	12	0·413	0·046	0·142	3·11	10	S.	0·432	0·048	0·123	2·589	10	S.
	fish	7	0·820	0·253	0·264	1·04	5	N.S.	0·622	0·206	0·066	0·421	5	N.S.
II	*Macaca* spp.	75	0·184	0·025	0·372	15·01	73	S.	0·264	0·035	0·291	8·27	73	S.
	Sciuridae	35	0·596	0·041	0·040	0·983	33	N.S.	0·641	0·016	0·086	5·30	33	S.

Table 2. Estimates of α for adult animals within the same species

set	species	number in species	estimate of α	weighted logarithmic method					standard logarithmic method					
				standard error of estimate	$\lvert0\cdot26-\alpha\rvert$	t	f	significance of t (5% level)	estimate of α	standard error of estimate	$\lvert0\cdot26-\alpha\rvert$	t	f	significance of t (5% level)
III	*M. rhesus*	53	0·102	0·035	0·158	4·529	51	S.	0·121	0·040	0·139	3·442	51	S.
	S. carolinensis	17	0·283	0·227	0·226	0·100	15	N.S.	0·279	0·105	0·194	0·100	15	N.S.
	M. nemestrinus	8	0·088	0·083	0·172	2·069	6	N.S.	0·173	0·051	0·087	1·711	6	N.S.
	M. cynomolgus	9	0·017	0·030	0·243	8·218	7	S.	0·163	0·139	0·097	0·698	7	N.S.
					$\lvert\alpha-0\rvert$							$\lvert\alpha-0\rvert$			
III	*M. rhesus*	53	0·102	0·035	0·102	2·940	51	S.	0·121	0·040	0·121	2·989	51	S.
	S. carolinensis	17	0·283	0·227	0·283	1·248	15	N.S.	0·279	0·105	0·279	2·651	15	S.
	M. nemestrinus	8	0·088	0·083	0·088	1·058	6	N.S.	0·173	0·051	0·173	3·424	6	S.
	M. cynomolgus	9	0·017	0·030	0·017	0·589	7	N.S.	0·163	0·139	0·163	1·177	7	N.S.

For brevity I shall list the hypotheses which I propose to test.

Hypothesis I. For sets I and II the value of the parameter α is not significantly different from $\frac{2}{3}$, i.e. $\left|\frac{2}{3} - \alpha\right| = 0$.

Hypothesis II. For set III the parameter α is not significantly different from 0·26, i.e. $\left|0 \cdot 26 - \alpha\right| = 0$.

Hypothesis III. For set III the parameter α is not significantly different from zero.

Hypothesis IV For sets I and II the values of log b are integral multiples of $\sqrt{2}$.

These hypotheses will now be considered in the light of the results shown in the tables.
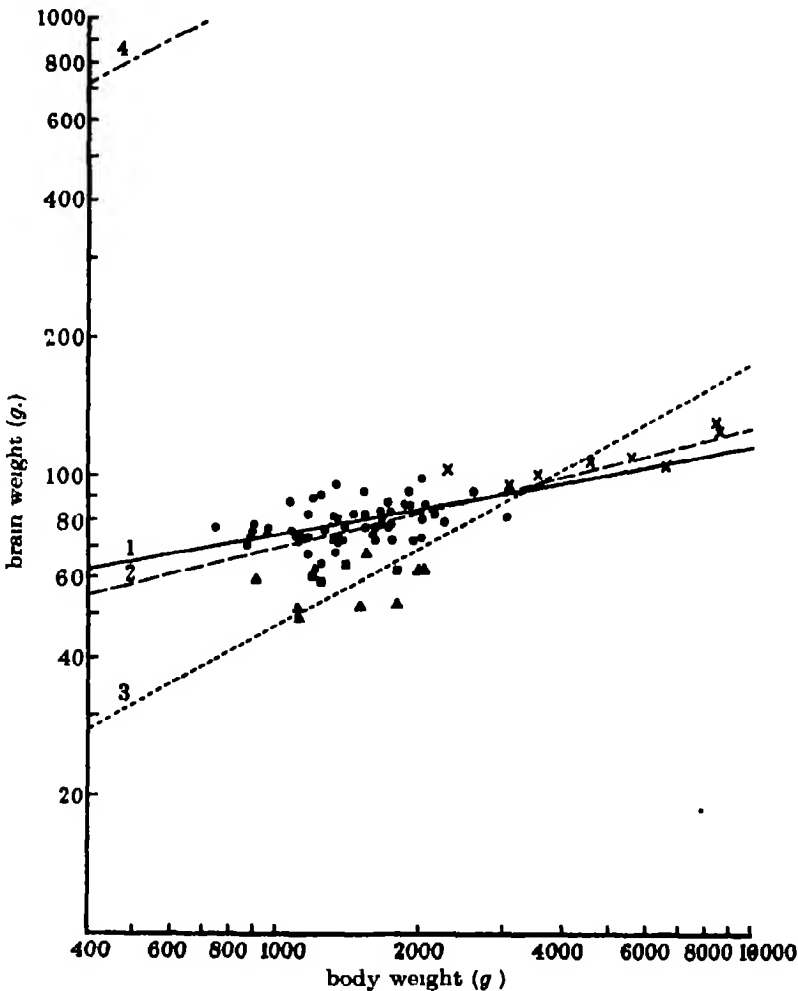
FIGURE 1. Logarithmic plot of brain and body weights of *Macaca* spp. 1, line $y = bx^{\alpha}$ (w.l.m.); 2, line $y = bx^{\alpha}$ (s.l.m.), 3, Dubois-Brummelkamp line $y = (\sqrt{2})^{0}\, x^{0\cdot 56}$, 4, Dubois-Brummelkamp line $y = (\sqrt{2})^{1}\, x^{0\cdot 56}$. ● *M. rhesus*; ▲ *M. cynomolgus*; × *M. nemestrinus*; ■ *M. pileatus*.

TABLE 3. ESTIMATES OF b

		weighted log method		standard log method	
group or species	number	estimate of b	standard error of estimate	estimate of b	standard error of estimate
1. rodents	32	0·118	0·032	0·062	0·090
2. ungulates	56	0·006	0·004	0·554	0·344
3. birds	25	0·691	0·142	0·116	0·040
4. Amphibia	12	0·022	0·044	0·019	0·003
5. fish	7	0·002	0·005	0·003	0·005
6. *Macaca* spp.	75	20·890	3·924	10·962	2·865
7. Sciuridae	35	0·197	0·049	0·150	0·014
8. *M. rhesus*	53	37·876	9·645	32·656	3·042
9. *S. carolinensis*	17	7·583*	10·556	1·357	0·876
10. *M. nemestrinus*	8	112·93*	80·419	25·397	10·933
11. *M. cynomolgus*	9	58·492*	12·388	57·234*	57·697

* Calculated on the assumption that α is not significantly different from zero
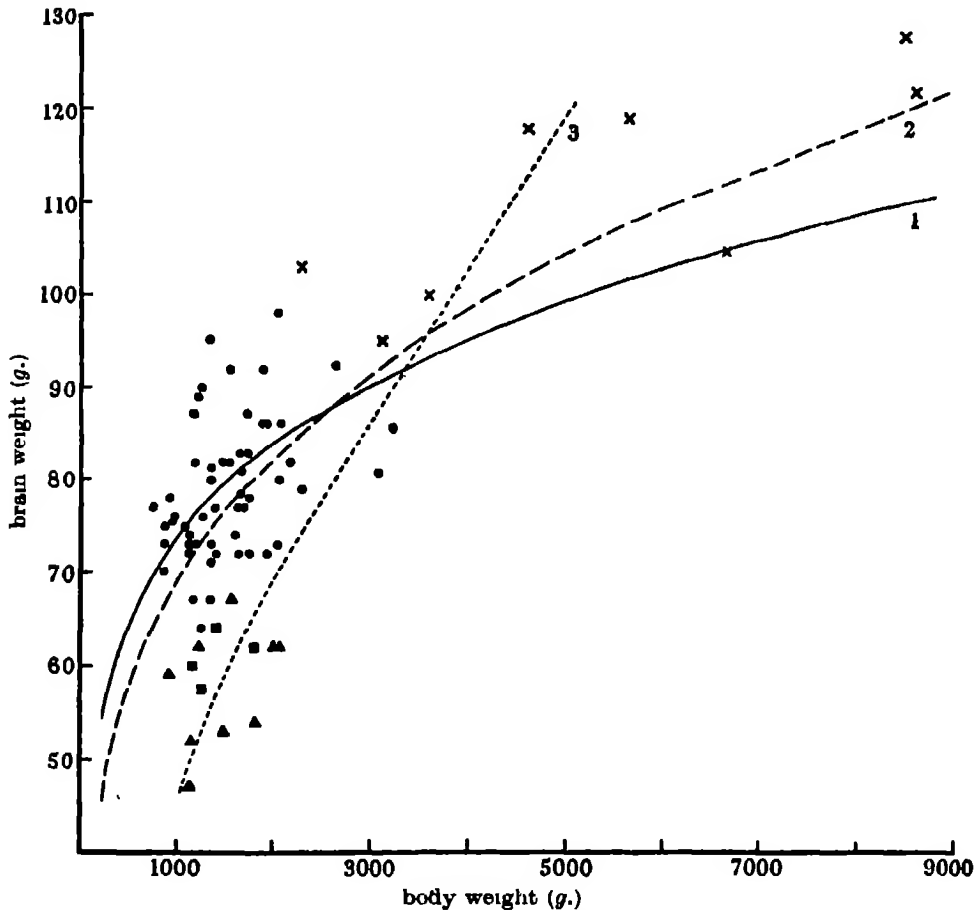
FIGURE 2. Plot of actual brain and body weights of *Macaca* spp. 1, line $y = bx^\alpha$ (W L M); 2, line $y = bx^\alpha$ (S.L.M.), 3, nearest Dubois-Brummelkamp line $y = (\sqrt{2})^\circ x^{0.56}$. (Key to points as for figure 1.)

Hypothesis I

The only group of data which does not reject the hypothesis by either method is that for fish. Here there were only seven observations with high variance, i.e. the scatter of the points is so great and the data is so rough that they would conform to a wide range of hypotheses. The hypothesis is not rejected by the s.l.m. applied to ungulates and birds.

However, in general, the hypothesis must be rejected, no matter which method of fitting is used, for the hypothesis with which we are concerned is stated to be true for all the sets, and the rejection of it by even one set would mean that the hypothesis could not be maintained.

Hypothesis II

Here, again, the hypothesis must be rejected although the data, such as they are, from some species do not reject it—the relatively high standard errors are again noticeable.

Hypothesis III

During the course of this work it occurred to me that it might be reasonable to suppose that the brain weight of an adult animal might have a constant value within a species, i e independent of the body weight Now, if $\alpha = 0$, then x^α would be unity and our equation would reduce to

$$y = b.$$

If we test that $\alpha = 0$, we find that it is rejected by all these species if the s.l m. fitting is used and by one if the w l.m. is used. We cannot accept this hypothesis on the data presented, nevertheless some points of interest arise and show that it is most necessary to decide whether we consider y or $\log y$ to be normally distributed. If we follow Reeve (1940) and assume that $\log y$ is normally distributed, then we should conclude that we have examples of the allometric growth of the brain in the few species considered here On the other hand, the assumption that y is normally distributed would lead to the conclusion that only in *M. rhesus* does the brain grow allometrically and that in the other three species there is a limit to the size of the brain and this is independent of the body weight. It would be very rash to draw definite conclusions from such data, but it is clear that the assumptions made in fitting affect these conclusions profoundly These results may be compared with those obtained in a different connexion by Zuckerman & Fisher (1937)

Hypothesis IV

This hypothesis is somewhat different from the others since it does not lend itself to statistical treatment in its original form It depends on an arbitrary grouping and, in fact, Brummelkamp merely says that if you plot the lines he suggests, the data group themselves about these lines. Count (1947) has discussed this point fully. In any case, this hypothesis assumes the truth of hypothesis I, and since that has been rejected this must also be rejected.

DISCUSSION

The results show that no matter which assumption is made as to the distribution of the variables, i.e. the variables themselves have a normal distribution or their logarithms are normally distributed, the data do not support the views put forward by the Dubois-Lapicque-Brummelkamp school. This does not imply that the allometry concept cannot be applied to the brain, but it does imply that the parameters do not have the specific values which have been maintained, i e. the evidence is against the hypotheses that the value of $\alpha = \frac{5}{9}$ for the interspecific groups or $\alpha = 0.26$ for animals of the same species. Count (1947) in his criticism of this theory fails to notice that the parameter estimates should be made by some recognized statistical method. Moreover, he proposes a relationship of the form

$$y = ax^{b-c\log x}. \tag{6}$$

A further point arises here   It will be noticed that (6) has three parameters, a, b, c. No matter how remote the mathematical model may be from the biological facts, the more parameters which are put into a formula the closer that formula will tend to fit the data, but we should still have to find a biological meaning for those parameters. Moreover, apart from this difficulty, it would have to be shown that the formula with the additional parameters does actually fit the data more closely, i.e. that it does represent the relationship between the observations more exactly. There are methods by which this could be tested, but Count does not appear to have availed himself of them, and no reasons are adduced for believing this formula to be 'better' than any other.

No theoretical justification is given for this formula and no matter what criticisms may be levelled against the manner in which the allometry formula has been used, it is possible to provide some such basis for it. The value of fitting empirical formulae to data has often been discussed and the failure to realize that a satisfactory fit of the formula to the data is no criterion for judging the 'truth' of the formula is still prevalent. Feller (1939) has made this very clear   Count states that 'other curves were unsatisfactory' but he makes no examination of the closeness of fit of these curves, nor does he show that, in fact, his curve fits the data any better than the simple allometric form. Again, Count considers that the fitting of his curve is precisely the same as fitting

$$\log y = \log a + b \log x - c(\log x)^2,$$

and consequently the main criticism of this paper would also apply, i.e. it is necessary to consider whether the variables or their logarithms are normally distributed, since the estimates of the parameters will vary with such assumptions.

Brummelkamp has extended his argument to embrace taxonomic problems. Reduced to its simplest form this would seem to mean that since, by hypothesis, the value of α is known, we can determine the value of b for any given species and if we then wish to decide the 'evolutionary stage' of an unknown animal we need

only to determine the value of b for it and then, since we have the b's of other animals for comparison and we have the relationship between the b's we can place our unknown animal in its position in the evolutionary scale. In the case of human beings, Brummelkamp transforms from brain and body weights to skull capacities and body lengths, but his argument rests on the constancy of the 'phylogenetic constant', etc., and we have shown that these hypotheses are untenable. If one could establish that the intraspecific value of α was always zero—and this would seem to be a common-sense point of view—then it might be possible to develop a method for taxonomic decisions. In any case, I am of the opinion that there would be a large possibility of error, and if it is desirable to make taxonomic decisions on brain and body-weight measurements, it would be much more profitable to use Fisher's method of discriminant analysis (Fisher 1936, 1938, 1941), which enables us to find a function of observations on related species which will make the assignment of an animal to its correct species possible.

Finally, while it may be possible to show that the allometric equation gives a satisfactory formulation of the brain/body relationship from the point of view of tissue masses, it would seem that it is futile to expect that one or both of the parameters will give any measure of intellectual development. The same criticism would apply to Count's formula or to some other formula which might relate body weight to some function of the weights of parts of the brain. Any development of this kind fails to take into account the fact that it is not how much brain an animal has, but what kind of brain it is that is of importance When the homologies of the parts of the brain are better understood it may be possible to make progress along these lines.

CONCLUSIONS

1   If it is considered that x and y are connected by the relationship $y = bx^\alpha$, the estimates of the parameters b and α found by the method of maximum likelihood will be different from those found by the same method if the relationship

$$\log y = \log b + \alpha \log x$$

is used as the basis of estimation.

2   The fitting of the relationship $y = bx^\alpha$ to data involves prohibitive calculation if more than a dozen observations are considered and even then only estimates of minimum variance can be found   It is shown that this relationship is equivalent to minimizing $\Sigma y^2 (\log y - \log b - \alpha \log x)^2$, and that, since this form is linear in the parameters their estimates and their standard errors can be found quickly.

3. The method has been applied to the data published by Brummelkamp and also to other published data and it is shown that no matter whether the form

$$y = bx^\alpha \quad \text{or} \quad \log y = \log b + \alpha \log x,$$

is assumed to apply to the figures, the hypothesis, that the inter-specific value of α is $\frac{2}{3}$ and the intra-specific value is $0\cdot26$ must be rejected. A fortiori the hypothesis that $\log b$ is an integral multiple of $\sqrt{2}$ cannot be accepted.

4. In certain species it is shown that the size of the adult brain may be independent of the body weight.

5. The concept of 'cephalization' is not well defined and a more profound quantitative study of the embryology and comparative anatomy of the brain is necessary before an adequate quantitative theory of the development and evolution of the brain can be formulated.

APPENDIX

Since the fitting of a curve of the form

$$y = bx^c$$

leads to considerable computation difficulties we shall consider an alternative approach.

Suppose that
$$y = f(x, a, b, c, \ldots),$$

where $f(x, a, b, c, \ldots)$ is a continuous function of x, a, b, c which possesses continuous partial derivatives and that η_i ($i = 1, 2, \ldots$) are observed values of y. Then we wish to minimize

$$\sum_i [f(x, a, b, c, \ldots) - \eta_i]^2, \tag{A}$$

and we find
$$\sum_i (y_i - \eta_i) \frac{\partial f}{\partial a} = 0, \quad \sum_i (y_i - \eta_i) \frac{\partial f}{\partial b} = 0, \quad \text{etc.} \tag{A i}$$

Now, let
$$\phi(y) = a u_1(x) + b u_2(x) + \ldots,$$

and suppose that
$$\sum_i \omega_i [\phi(y_i) - \phi(\eta_i)]^2$$

has a minimum at the same point as (A). Then

$$\sum_i \omega_i [\phi(y_i) - \phi(\eta_i)] \frac{\partial \phi(y)}{\partial a} = 0,$$

i.e.
$$\sum_i \omega_i [\phi(y_i) - \phi(\eta_i)] \frac{\partial f}{\partial a} \frac{\partial \phi}{\partial y} = 0 \tag{B}$$

Further, since $\phi(y_i) - \phi(\eta_i) = (y_i - \eta_i) \phi'(\eta_i) + \frac{(y_i - \eta_i)^2}{2!} \phi''(\eta_i) + \ldots$,

and we may write (B) in the form

$$\sum_i \omega_i [(y_i - \eta_i) \phi'(\eta_i)] \frac{\partial f}{\partial a} \frac{\partial \phi}{\partial y} = 0, \tag{C}$$

and we require (A i) and (C) to be identical, i.e.

$$\sum_i (y_i - \eta_i) \frac{\partial f}{\partial a} = \sum_i \omega_i (y_i - \eta_i) \frac{\partial f}{\partial a} \phi'(\eta_i) \frac{\partial \phi}{\partial y}.$$

This will be the case if
$$\omega_i \phi'(\eta_i) \left(\frac{\partial \phi}{\partial y} \right) = 1,$$

and provided the expansion remains valid

$$\omega_i\left(\frac{\partial\phi}{\partial y}\right)^2_{y=y_i} = 1.$$

In our case $\phi(y) = \log y$ and so $\omega_i = y_i^2.$

This is the origin of the factor y^2 in equation (5) of the main part of the paper. Iteration of the computation may improve the accuracy of the estimates.

My first demonstration of this approximation was clumsy, and I am very much indebted to Mr W. Rudoe for the elegance of the demonstration given here and for much discussion on the mathematical points involved

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The Wellcome Research Institution

By C. H. Kellaway, F.R.S., *Director-in-Chief*

[Plates 14 to 17]

The Wellcome Research Institution is the name which Sir Henry Wellcome used to describe collectively the Museums and the various research undertakings of the Wellcome Foundation Ltd. Before outlining the development of the individual units I must first briefly relate the history of the Wellcome Foundation itself.

The firm of Burroughs Wellcome and Co. was founded in London in 1880 by two young American pharmacists, Silas M. Burroughs and Henry S. Wellcome. This proved a most successful undertaking, and after the death of Burroughs in 1895 Wellcome became the sole owner of a flourishing business with commercial offices in Snow Hill, chemical works at Dartford and the beginnings of his earliest research enterprise, the Wellcome Physiological Research Laboratories.

The next 30 years saw the development not only of the business in this country but of associated houses in U.S.A. and other parts of the world, and in 1924 Wellcome consolidated all his interests in a single company, the Wellcome Foundation Ltd. This fusion was done with a deliberate purpose later to be revealed by his will. Within the framework of the business itself he had already developed the laboratories for scientific research and the educational museums that I shall presently describe. Beyond these the will provided for scientific work outside the business itself, for, on Wellcome's death in 1936, all the shares in this Company were vested in five trustees who were to receive the whole of the distributable profits and use them in certain specified ways for the advancement of research in medicine and its related subjects anywhere in the world.

The Wellcome Physiological Research Laboratories

When diphtheria antitoxin first came into use Mr Wellcome realized its importance and in 1895 set up laboratories in London for its manufacture. In 1898 these were moved to Brockwell Hall, Herne Hill, which, with 10 acres of ground, stabling and other buildings, provided suitable accommodation not only for the manufacture of the new serum but for the more ambitious research projects Mr Wellcome had in mind. These were foreshadowed by the name he gave the laboratories in 1895.

The research history of this unit did not commence until the beginning of the present century, when, in spite of opposition by the Royal Colleges of Physicians and Surgeons, the premises at Brockwell Hall were registered as a place where experiments on living animals might be performed. This was the first instance of such recognition for laboratories attached to a commercial firm.

During 52 years this unit has had three periods of active research interrupted by two world wars, during each of which nearly all research, except what was essential to production, ceased. During the first period from 1900 to 1914 the laboratories

were directed first by Dr Walter Dowson till 1906 and then by Dr H. H. Dale till the outbreak of the first world war.

Dr Walter Dowson, though not himself an experienced scientific worker, was a capable administrator who succeeded in attracting young and well-trained scientific personnel and in creating good conditions of work for them. Among the early members of the scientific staff were a number of men who subsequently became Fellows of this Society—George Barger, H. H. Dale, A. J. Ewins, A. T. Glenny, P. P. Laidlaw and John Mellanby, and at a somewhat later period, Harold King and J. H. Burn.

John Mellanby was the first pharmacologist appointed to the laboratories and spent several years there in studying the globulins and other proteins from horse plasma and serum. He described a method for concentrating diphtheria antitoxin without denaturation of the protein by treating the serum with alcohol at low temperatures—a principle which has recently been brilliantly developed and extended by Cohn and his colleagues at Harvard.

A. T. Glenny, who only last year retired from the staff, controlled the production of antisera at the laboratories for about 40 years but found time to utilize for research the wealth of material which was available to him as well as to carry out well-planned experiments on immunity.

The association of Barger, Dale, Ewins and Laidlaw during these early years was a most fruitful one. When Dale joined the staff as pharmacologist in 1904 Barger had already commenced the investigation of ergot which not only yielded valuable direct results but led to studies of even greater importance The alkaloid, ergotoxine, was discovered through the recognition of its curious action of adrenaline reversal. Histamine, which in the intervening years has proved to be of great interest in relation to anaphylaxis and various shock-like conditions and in the immediate reaction to injury, was isolated from extracts of ergot and its pharmacological action was analyzed by Dale and Laidlaw. Other amines also engaged attention, and Dale and Barger made an extensive study of the relation of their chemical structure to sympathomimetic action.

The action of ergot in causing strong contraction of the uterus led Dale to the study of another powerful oxytocic present in extracts of the posterior lobe of the pituitary gland. This work formed the basis for the method of standardizing such extracts described by Dale and Laidlaw which is still in general use.

The observation that a particular ergot extract had peculiar properties led to the identification in it of acetylcholine by Ewins. The discovery by Dale of the muscarine- and nicotine-like activities of this substance, by which it simulated effects of different groups of efferent nerves, provided the foundation for studies later resumed by Dale and his colleagues at the National Institute; these, with the researches of Otto Loewi, played a formative part in the modern conception of the chemical transmission of nervous stimuli.

Other important researches were the examination of the anaphylactic response of the uterus of the sensitized guinea-pig by Dale and investigations of the hydrogen-ion concentration of biological fluids by G. S. Walpole, who first organized the concentration of antitoxin at the laboratories.

The scientific staff at this time was small, less than a dozen, but they were a very active group, and during this period about 100 papers, many of them of classical importance, were published.

The year immediately preceding the outbreak of the first world war broke up this happy company. Laidlaw went to the professorship of pathology at Guy's and Dale joined the research team which was being built up by the Medical Research Council, taking with him Barger and Ewins. Dale was succeeded by Dr R. A. O'Brien who, with a greatly reduced scientific staff, was confronted by the wartime problem of a large increase in production. During the war the production demand was fully met and large quantities of tetanus and diphtheria antitoxins, antigangrene sera and typhoid vaccines were made available for the forces.

Shortly after the war Mr Wellcome bought a property of rather more than 100 acres at Langley Court, Beckenham (figure 2, plate 14), and the move there was made in 1922. During the period between the two world wars the scientific staff gradually increased in number, reaching a total of 28 in 1939. Of the staff in this interwar period three more were later to become Fellows of this Society—Percival Hartley, J. H. Gaddum and J. W. Trevan.

Between 1921 and 1939 over 300 papers were published. Glenny now had his most productive period. He had already discovered the difference in effect between primary and secondary stimuli in active immunization, though the publication of this work had been delayed, and he now contributed year by year a series of papers on active and passive immunization mainly concerned with the response to diphtheria toxin. With Pope and Miss Hopkins he was early in the field in the conversion of toxin to toxoid by formaldehyde, and he and his colleagues discovered the advantageous effect of precipitating toxin with alum. Alum-precipitated toxoid (A.P.T.), and indeed other alum-precipitated toxoids, have since been very widely used for immunization.

O'Brien was the first in this country to carry out Schick testing and was a pioneer advocate of active immunization against diphtheria. Dalling's work contributed much to the control of lamb dysentery, a disease which formerly killed many thousands of lambs each year in Britain. He identified the causative organism and showed that the pregnant ewe could be actively immunized by vaccination and could transmit effective immunity to its offspring. With Glenny he elaborated an antitoxic serum for the passive protection of newly born lambs.

Hartley during 1920 and 1921 developed a medium for the production of diphtheria toxin which has found worldwide use and made possible uniform production of potent diphtheria toxin. This work has been the basis of the great improvements in toxin production which have been made since that time. He was also a pioneer in the rapid drying at low temperatures of sera and other protein solutions, developing a method which he found most useful during the next 25 years when he was in charge of the Standards Laboratory at the National Institute for Medical Research.

Dalling made generally available the method of immunization against dog distemper which has been developed by the researches of Laidlaw and Dunkin at the National Institute for Medical Research.

Pope made an important contribution in developing a method for concentrating antitoxic sera which depended upon preliminary digestion and heat denaturation of unwanted proteins. The use of this and other methods of serum concentration has greatly diminished the incidence of serum sickness after the therapeutic use of antitoxin.

During this period much attention was directed to accurate methods of standardization of therapeutic substances, and Trevan, in 1927, published an important paper on the statistical basis of determinations of the lethal dose and other biological end-point reactions, which has had a wide influence on the design of methods of bio-assay.

During the period immediately before the second world war Buttle and his associates were actively engaged in studies on chemotherapy, particularly on sulphone derivatives and sulphonamide.

During the early part of the recent war, under the direction of Dr R. A. O'Brien and after 1941 under Dr J. W. Trevan, the laboratories were faced with an even larger production demand than in the first world war. Vast quantities were made and issued of tetanus and gas gangrene antitoxins, tetanus toxoid, and the major part of the diphtheria prophylactics used by the Ministry of Health in its free immunization campaign Additional laboratory accommodation was needed and the L.C.C. made available their laboratories at Carshalton and Belmont. Over 1000 horses had to be maintained for the manufacture of sera and stabling accommodation was acquired in various parts of the country, some even so far afield as Doncaster. When the Veterinary Research Station was established at Frant, in Sussex, in 1943 some further stabling accommodation had to be provided there.

During the latter part of the war a large mechanical surface culture plant was set up at Beckenham for the manufacture of penicillin, but in 1945, when it became apparent that manufacture by the bottle process could not compete economically with deep culture, its use was discontinued. Before it was closed down some streptomycin was produced for experimental purposes in a portion of the plant. Work on antibiotics was now confined to search for new ones in a small unit at Belmont, which had been used for the manufacture of penicillin, and later in the main laboratories at Beckenham.

At the end of the war diminished production again made possible increased research activity and there has been a large increase in the number of papers issuing from these laboratories The most important recent work of which mention should be made are further studies on immunity, on the toxins of the *Cl. welchii* group, on the pharmacology of 'Sulphetrone' and streptomycin, work on the production of diphtheria toxin by deep culture, the development of a new method of refinement applicable to antibacterial sera which depends on precipitation with ammonium sulphate in the presence of tricresol, and studies on 'Aerosporin', a new antibiotic which covers part of the bacterial spectrum not adequately covered by either penicillin or streptomycin. Aerosporin appears not readily to allow of the production of resistant bacterial strains, and gives promise of real value in the treatment of whooping cough and of some other diseases caused by Gram-negative organisms.

THE WELLCOME VETERINARY RESEARCH STATION

By the end of 1943 it had become apparent that the property at Beckenham was not adequate for the satisfactory breeding of the large number of small animals needed for testing and research, and a farm of more than 300 acres was purchased at Ely Grange, Frant (figure 7, plate 17). Here small animals could be bred under isolated conditions, particularly ferrets for developmental research on distemper products. Here too the green food needed for stock animals and those under experiment at Beckenham could be grown and a suitable place was provided for the investigation of diseases of larger animals, particularly of sheep and cattle. The scientific staff, under the direction of Dr R. F. Montgomerie, has ranged from three to six in number and the chief problems so far studied there have been mastitis in milking herds, bovine infertility, problems connected with small animal breeding and the study of immunity against louping ill. At the end of 1946 a temporary Artificial Insemination Centre was set up there in collaboration with the Milk Marketing Board.

The smooth development of the Frant farm as a veterinary research station suffered somewhat from the war enterprise given the secret name of the 'Tyburn' operation, which was carried out there in 1945. By the end of 1944 it had become apparent that scrub typhus might be a serious menace to jungle operations in south-east Asia and elsewhere, and the War Office decided to have made on a large scale the scrub typhus vaccine which had been devised by Fulton and Joyner at the National Institute for Medical Research using the lungs of cotton rats infected with the causative rickettsiae. The Ministry of Supply with the advice of the Medical Research Council requested the Wellcome Foundation to undertake this task and the Medical Research Council seconded to us Dr M. van den Ende to take charge of the scientific side of the operation and direct the developmental and other researches which were carried out during its progress. The manufacture of the vaccine involved the importation by air from America of thousands of cotton rats and the simultaneous breeding of large numbers of these animals in this country. Ely Grange, Frant was selected for the enterprise as being sufficiently isolated and as providing accommodation for the workers engaged in a difficult and dangerous project. The scientific personnel, nine in number, were partly civilian and partly officers of the R.A.M.C. and the A.T.S. and the sixty technical personnel were R.A.M.C. laboratory technicians and A.T.S. who volunteered for the work.

The laboratories were built in 109 days and the preparation of vaccine was started on 1 May 1945. Between that date and 31 October, when manufacture was discontinued, 300 l. of vaccine, enough to vaccinate 100,000 men, were made. This was a highly dangerous technical operation involving inoculation of cotton rats by insufflation with a seed suspension of rickettsiae from the lungs of mice infected in the same way. The rats were kept for several days and died or were killed, the lungs being harvested and emulsified to make the vaccine which was treated with formalin to kill the rickettsiae. The workers engaged in the manufacture were vaccinated and the most dangerous operations were carried out in cabinets ventilated by suction, the air from these being sterilized by special furnaces on the roof

of the laboratories. A good many other precautions were used—the air in the laboratories was treated by aerosols of lactic acid and very carefully controlled procedures were laid down to minimize risk. Despite these precautions, four workers were accidentally infected. All had, of course, been protected by vaccination and fortunately made uneventful recoveries.

THE WELLCOME CHEMICAL RESEARCH LABORATORIES

These Laboratories were founded in London in 1896 and remained at 6 King Street, Snow Hill for 35 years. In 1923 they were transferred to the new building of the Wellcome Research Institution at 183 Euston Road where a portion of the staff is still housed as part of the Laboratories of Tropical Medicine (figure 5, plate 16).

When the Laboratories were founded, Mr Wellcome persuaded his friend, F. B. Power, to come over from America and take charge of them. During Power's directorship work was mainly concentrated on increasing systematic knowledge of the chemical constituents of plants used in medicine, and a wide range of these was investigated. During this period in the development laboratories at Dartford Dr H. A. D. Jowett carried out his work on the constitution of pilocarpine which was shown to contain a glyoxaline nucleus, Dr F. L. Pyman synthesized two other physiologically important glyoxaline derivatives, histidine and histamine, and Jowett and Pyman did their work on the tropine esters.

When Pyman followed Power as Director of the Chemical Research Laboratories in 1914 attention was focused on synthetic drugs and alkaloids. Pyman continued the fruitful studies commenced at Dartford on the ipecacuanha alkaloids. Dr Harold King carried out his important work on the resolution of the optically active components of hyoscine. The Laboratories during the first world war were also faced with the problem of devising means of replacing German synthetic drugs, the most important of which was arsphenamine.

When Dr Henry became Director in 1919 the main interest of the Laboratories was turned towards the chemotherapy of tropical disease. Some synthetic compounds were prepared for the treatment of hookworm and the investigation of chenopodium oil which was being extensively used for this purpose showed that its only active component was ascaridole. Work on the antimony compounds for the treatment of kala azar resulted in the synthesis of the N-glucoside of p-aminophenyl stibonic acid ('Neostam'). Just before the second world war a new pentavalent antimony compound was devised in Germany for the treatment of kala azar, and early in the war the composition of this drug was determined and a process designed for its manufacture.

Work on antimalarials included an extensive investigation of the alkaloids of the genus Alstonia and a study, carried out on behalf of the Malaria Commission of the League of Nations, of the composition and standardization of the quinine alkaloids.

The 4-4'-diaminodiphenylsulphone derivative, 'Sulphetrone', which shows promise in the treatment of leprosy was also synthesized in these laboratories.

Meanwhile at the Development Laboratories at Dartford, under Dr Sydney Smith's direction, research on ephedra led to the isolation of two new alkaloids.

Here too Dr Smith isolated digoxin, the pure crystalline glucoside of *Digitalis lanata*.

When Dr Henry retired in 1944 Dr Smith took charge of the main laboratories which were to be established at Beckenham when the new building (figure 6, plate 16) was completed. In the meanwhile accommodation was found for Dr Smith and three of his colleagues in one of the more modern laboratories at Langley Court where this team worked on penicillin synthesis making an important contribution in the isolation of penillic acid. The move into the new building took place in 1946, a portion of it being used to house part of the chemotherapeutic testing unit.

The new laboratories have been working for too short a period for more than the most general report of their activities to be made. A number of new phenanthridinium compounds, active against more than one species of trypanosome, have been synthesized, interesting studies have been made on the chemistry of the new antibiotic, Aerosporin, and work on substitutes for *d*-tubocurarine and on analgesics and antiasthmatics has been in progress.

THE WELLCOME LABORATORIES OF TROPICAL MEDICINE

Mr Wellcome's first venture into tropical research was conducted to some extent vicariously. In 1901 he offered to the Gordon Memorial College, Khartoum, equipment for chemical and bacteriological laboratories, the College being responsible for the building and for the selection and support of the scientific staff. The Wellcome Tropical Research Laboratories so founded continued until 1935 when they were closed down by the Sudan Government, the space occupied by them being needed for other purposes. The first Director of these Laboratories was Dr Andrew Balfour. In 1907 Mr Wellcome provided a floating laboratory to carry research to regions which were otherwise not readily accessible and Dr C. M. Wenyon was placed in charge of this.

In 1913 the Wellcome Bureau of Scientific Research was founded with Andrew Balfour as Director and as Director-in-Chief of all the various research units which Mr Wellcome had set up. The Bureau was to be a centre in London for the study of tropical disease with the important function of supplying information to workers abroad, and its staff included C. M. Wenyon as protozoologist, A. C. Stevenson as pathologist and M. E. MacGregor as entomologist.

Shortly after the Bureau had been established the first world war broke out and the Bureau was placed at the disposal of the War Office. It played an important part in training medical officers in tropical disease and Dr Balfour wrote the well-known 'Memoranda on Medical Diseases in the Tropical and Sub-Tropical War Areas'. Balfour and Wenyon spent most of the war years at various fronts mainly acting as consultants; and Wenyon did his important work on amoebic dysentery in Egypt and on malaria in Macedonia.

During the war the development of the Bureau was necessarily inhibited, but when the war ended its staff was increased and in 1920 it moved from Henrietta Street to more commodious premises at Endsleigh Gardens. From this time till the present it has had generally 9 to 11 senior scientific workers on its staff.

In 1923 Balfour resigned, becoming Director of the newly-created London School of Hygiene and Tropical Medicine, and Wenyon succeeded him as Director of the Bureau of Scientific Research and Director-in-Chief of the Wellcome Research Institution. The policy was followed of seconding workers to various laboratories abroad; for example, in 1927 C. A. Hoare went to work on trypanosomiasis in the Sleeping Sickness Laboratory at Entebbe, and later G. M. Findlay made numerous ventures in different parts of the world to study yellow fever and other diseases. The Bureau was also available for guest workers, and it is of interest that in 1928 Dr E. Hindle began there his work on yellow fever for the Yellow Fever Commission of the Colonial Office, in which he carried out the first preventive vaccinations on monkeys with formalinized virus.

In 1932 the building in the Euston Road (figure 1, plate 14) was erected to house the Museums, the Bureau and the Chemical Research Laboratories. In their new quarters work was now begun on virus diseases, and on leptospiral infections. The manufacture of yellow fever vaccine on a laboratory scale was started by G. M. Findlay, who had visited the United States to study the methods of research and manufacture at the Rockefeller Institute. Numbers of service personnel were inoculated year by year and, when the second world war broke out, free inoculation was continued on a large scale subsidized to some extent by the Colonial Office until the end of the war.

The problem of infective hepatitis and of serum jaundice had in the intervening years engaged the attention of workers at the Bureau and during the war F. O. MacCallum was seconded to work at Cambridge on a special enquiry concerning infective hepatitis, financed by the Medical Research Council.

In 1944 Dr Wenyon retired and the departmental controls were reorganized. I joined the staff as Director-in-Chief and Dr N H. Fairley was invited to become Director of the Laboratories of Tropical Medicine and also professor in the new Chair of Tropical Medicine founded by the Wellcome Trustees at the London School of Hygiene and Tropical Medicine. Fairley, however, found himself so fully occupied by his professorial duties that he resigned and was succeeded late in 1946 by Brigadier J. S. K. Boyd who had just retired from the directorate of pathology at the War Office.

The Bureau of Scientific Research, or to use its present name, the Wellcome Laboratories of Tropical Medicine, during its 34 years existence has had a distinguished record in research in tropical disease, and 8 books and more than 560 papers have been contributed by members of its staff, the most important being C. M. Wenyon's classical *Manual of Protozoology* published in 1926.

The main problems on which work is now in progress concern bacteriophages, the tissue culture of protozoa, the serology of leptospirosis and the chemotherapy of tropical diseases (figure 4, plate 15).

The policy of sending workers abroad is being continued—Dr J. M. Watson having spent most of last year in Egypt studying bilharzia, a member of the Egyptian State Health Service having been an exchange guest worker in the laboratories here.

Closely associated with the Bureau are the Entomological Field Laboratories which were opened at Wisley in 1920 by Mr M. E. MacGregor. In 1921 the labora-

tories were moved to new premises at Esher in Surrey where for the last few years Mr B. Jobling has been carrying on his beautiful anatomical and histological studies on the blood-sucking diptera. At the present time when other large and well-equipped laboratories for entomological study are available in this country there seems little justification for the continuation of this small unit.

THE WELLCOME MUSEUM OF MEDICAL SCIENCE

The Museum of Medical Science arose primarily from the interest of Andrew Balfour in teaching museums. He had started such a museum at Gordon College and as soon as he took charge of the Bureau in 1913 he commenced to develop one there. The real history of the Museum began when Dr S. H Daukes, who had been appointed Curator in 1919, became Director of the Museum and in 1923 extended its scope to include diseases of temperate as well as tropical climates with the object of presenting an up-to-the-moment picture of advances in medicine.

The Museum grew rapidly and in 1932 three spacious galleries (figure 8, plate 17) were provided for it in the new building in Euston Road. Here Dr Daukes set it out in admirable fashion and secured a wealth of material illustrating the aetiology, symptomatology, pathology, treatment and prevention of disease. There were also sections on general and special pathology. The Museum was increasingly used by students, doctors, and nurses and others and in the year before the second world war began it had over 10,000 visitors.

After the outbreak of the second world war, when it appeared likely that London would be bombed, the Museum was dismantled and the valuable collection stored. In 1945, when this threat was removed, it was possible to re-establish it in part, despite considerable damage to screens and windows caused by neighbouring bomb explosions A beginning was made with the East wing, in which much of the tropical material is set up, and Dr Daukes was able to complete this before he retired at the end of 1945. He was succeeded by Dr C. J. Hackett, who, before continuing the establishment of the Museum, decided to modernize its presentation The system introduced by Daukes could not be bettered, but improvement in lighting, with re-arrangement enabling visitors more readily to grasp the essential features, will undoubtedly increase the value of this much-used collection. It will unfortunately be several years before the Museum is complete and up-to-date in every part.

THE WELLCOME HISTORICAL MEDICAL MUSEUM

The story of the development of this museum is really a part of the story of Henry Wellcome's life. In 1905, having arranged his affairs so as to obtain more leisure, he started collecting all kinds of material connected with the history of medicine, and the idea of creating a great museum of medical history became his keenest interest till the end of his life.

In 1913, he found housing for his collection in Wigmore Street, and displayed there much of his best material. The collection, which was used for a number of important exhibitions, remained there till 1932, when the new building in

Euston Road was ready for its reception. From 1924 onwards Sir Henry Wellcome devoted increasing time and energy to the collection of material. After the move to the new building the Museum was not at once opened, but the work of sorting the material was begun. Sir Henry Wellcome, who had just been elected a Fellow of this Society, had hoped finally to settle the layout, but he died in 1936 with his plan for this still uncompleted.

In his lifetime Sir Henry Wellcome himself was Director of this Museum and it was not till 1943 that Dr Daukes, who till his retirement in 1945 acted as Director of both Museums, proposed a plan for its re-arrangement when this could again be undertaken. At the end of 1945 this plan was in the course of being put into effect. In 1946 Dr E. Ashworth Underwood, a distinguished scholar of medical history, was appointed Director in succession to Dr Daukes and made plans to open not only the Museum but also the Library of 150,000 volumes, which till then had not been available for consultation, the work of cataloguing it being only about two-thirds completed.

Unhappily last year the Museum had a further and most disappointing setback, which will delay for some years its establishment in the building in which Wellcome had planned to house it. In 1940 when the central offices of the business at Snow Hill were completely destroyed it was necessary that its administrative staff should be given immediate accommodation within the building in Euston Road. This was not a serious matter during the war, since considerations of safety of the valuable material limited the use of the galleries as a Museum. In due course premises were found on short lease for housing most of the business and administrative staff, in the expectation that at the end of the war new accommodation could be built to .take the place of the offices destroyed by enemy action.

When in 1947 the temporary lease of these premises came to an end, prolonged and active search during two years through the whole of London for suitable premises having failed and the prospect of building new offices being removed to an uncertain future, nothing remained but to house the business activities in the building in Euston Road and to put most of the Museum material into store, until such time as the galleries once more become available for its display.

Fortunately, however, the Wellcome Trustees have been able to secure accommodation to house Dr Underwood and the Museum staff, and to afford them opportunity to continue their researches, as well as to give limited public exhibitions of valuable parts of the collection from time to time. The Library, though not ideally set up, remains in the Euston Road building and will be available for consultation by scholars interested in the history of medicine

Having now traced the history of this rather complex research organization which Henry Wellcome built up it remains to get its structure into perspective and to indicate the causes of its early success. Wellcome was an autocrat and during his lifetime rigidly controlled the organization in the directions he desired. He early recognized the importance of scientific research for the development of his business interests and in the process gained an enthusiasm for the application of science in all three of the ways Sir Edward Appleton has recently described, 'applied research, objective fundamental research and free fundamental research'. The scientific

workers whom he engaged were allowed to work largely on subjects of their own choice although he sometimes suggested topics in which he was particularly interested. They were also at liberty to publish the results of their researches and were thus not detached by rules of secrecy from their academic colleagues. This, fifty years ago, must have been an unusual situation to be occupied by men who had been attracted to industrial laboratories. The application of this policy was in the main responsible for the scientific distinction of the workers in the laboratories and for the high reputation of the laboratories themselves.

Wellcome's interest in education resulted in the development of the Museum and Library of Historical Medicine, the Bureau of Tropical Medicine and the Museum of Medical Science. The close collaboration of these last two units with the Tropical Diseases Hospital and the London School of Hygiene and Tropical Medicine has helped to make London an important centre for the study of tropical disease.

Though these educational units were within the framework of the business, Wellcome made some attempt to set them apart when he designed the great building in Euston Road to house them, but the second world war and its aftermath have prevented the smooth development of his large conception.

In the meanwhile the research organization must be directed towards increasing the prosperity of the business and the funds available to the Trustees for the broad purposes indicated by the Founder's will. In this country the Foundation has extensive and well-equipped laboratories and at the present time has a staff of about 80 graduate scientific workers, a large number of skilled technicians and many young men and women who are acquiring university degrees while they are engaged as technical assistants. This does not include the staff of the Chemical and Pharmaceutical Development Laboratories at the Works at Dartford who are responsible, under the direction of Dr Denis Wheeler, for the improvement of the processes in operation there as well as for development to the stage of production of projects handed over by the research staff. On the biological side there is no segregation of development and a good many of the scientific workers at the Beckenham laboratories are engaged in production of biological products for the improvement of which continuous developmental work is required.

As the research organization increased in size there was until recent years some tendency for units, and even for departments within units, to work independently. This has been discouraged and a degree of plasticity has been achieved which enables a number of workers to be concentrated on any urgent problem. Our current researches are co-ordinated with those being carried out in the research unit in our New York company and each part of the organization is being made aware of what is going on elsewhere. This important objective is being achieved by the circulation of regular reports on the progress of research projects, by visits between the units and by annual research meetings, attended by all the workers at which the current researches are reviewed and new projects indicated. In addition to these more general meetings the workers engaged in each project meet for frequent consultation and from time to time their projects are reviewed more formally, in the light of the current project reports, by myself and the heads of the research units concerned.

Publication remains free and is encouraged, and, though the bulk of work done is directed specifically towards the discovery of new therapeutic agents and therefore comes into the category of applied research, fundamental studies of the objective type form an important part of our programme.

FIGURE 1

FIGURE 2

FIGURE 3

FIGURE 4

FIGURE 5

FIGURE 6

FIGURE 7

FIGURE 8

Antiplasmodial action and chemical constitution
IX. Carbinolamines derived from 6:7-dimethylquinoline

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This communication completes this series of investigations. Ten carbinolamines derived from 6:7-dimethylquinoline and based on the quinine model are described. Three of the substances containing the 2-phenyl-6:7-dimethylquinoline nucleus show antiplasmodial activity on malaria in canaries much superior to the activity of quinine. The 6:7-dimethyl groups present in these compounds show a structural affinity with riboflavin but the antiplasmodial results are not favourable to the view that riboflavin-antagonism plays any significant part in the mechanism of action of these active bases.

In part II of this series (Ainley & King 1938) it was shown that 4-(6-methoxy-quinolyl)-α-piperidylcarbinol (II), a simple synthetic substance having many of the structural features of quinine (I), had one-half the activity of the latter on bird malaria. In part III (King & Work 1940) a still simpler structure (III), as exemplified by the dibutyl-, diamyl- and dihexyl-aminomethyl-6-methoxy-4-quinolylcarbinols, was found to carry antiplasmodial activity.
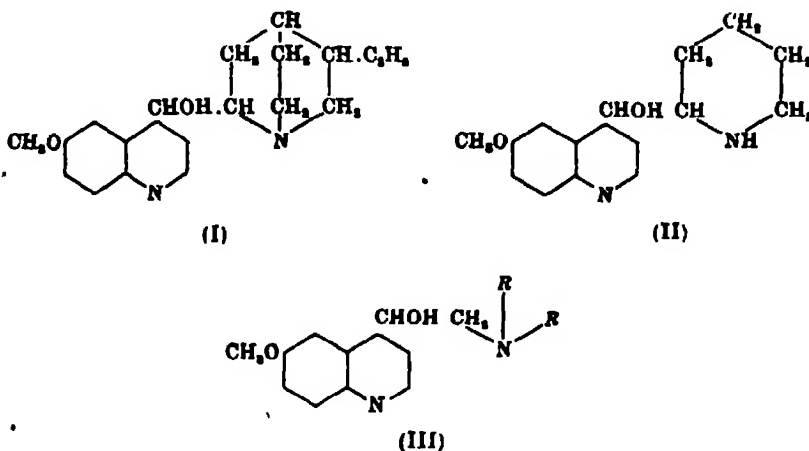
Two fundamental types were thus available for development in many directions, and during the War a great number of compounds, some of outstanding antiplasmodial activity, have been synthesized along these lines in the United States; a summary of their biological activities is given in *A survey of antimalarial drugs* (1946). It should be noted that these compounds were synthesized without invoking the general hypothesis on the mode of action of chemotherapeutic drugs formulated by Fildes and others. In fact, it is only within the last few years that any progress has been made in the biochemistry and metabolism of the many stages of the

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malarial parasite, so that the development of antagonists to essential metabolites has not hitherto been possible.

The mode of action of quinine on the malarial parasite is not known, but the findings of Laser (1946), who showed that many antimalarial drugs, including quinine, lower the *in vitro* rate of haemolysis by a haemolytic substance present in normal plasma, may enable a rational approach to be made to the devising of new antiplasmodial drugs when the constitution of this haemolytic substance has been elucidated.

On the other hand, atebrin (IV) bears a strong structural resemblance to riboflavin (V), and it has been suggested by Madinaveitia (1944, 1946) that the antiplasmodial action of atebrin may be due to an antagonism between the drug and one of the riboflavin-containing systems almost certainly present in the malarial parasite.

CH$_2$O ... NH CHMe CH$_2$ CH$_2$ CH$_2$ N(C$_2$H$_5$)(C$_2$H$_5$) ... N ... Cl

(IV)

CH$_3$ CH$_3$ ... CH$_2$ CHOH.CHOH.CHOH.CH$_2$OH ... N ... N ... O ... NH ... O

(V)
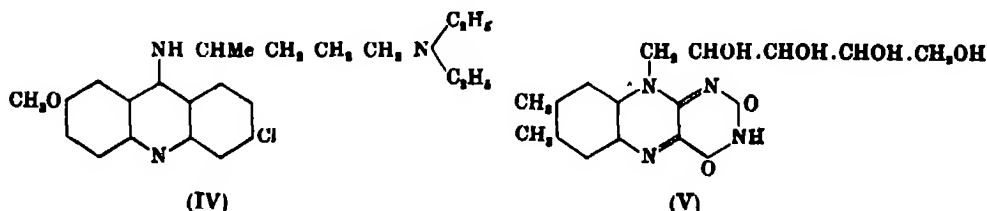
On this hypothesis attempts have been made by King & Acheson (1946) and others (*A survey of antimalarial drugs* 1946) to build *iso*alloxazines of the type of (V) with basic side-chains in the hope that such substances would have antiplasmodial properties. Some success has been attained along these lines.

Quinine, with its methoxylated quinoline nucleus and its elaborate basic side-chain (I), has much structurally in common with atebrin (IV), and it appeared to us that the action of quinine, in part at least, might be antagonistic to riboflavin-containing enzyme systems in the malarial parasite. This possibility being granted it was of interest to build a series of bases of the type of (III) containing a 6 : 7-dimethyl-quinoline nucleus with methyl groups structurally spaced as in riboflavin itself, and to compare the antiplasmodial properties of these with those of corresponding bases without the methyl groups but with the 6-methoxy group. If a eutherapeutic effect was produced by the two adjacent methyl groups, then the view that a part, at least, of the action of quinine and simpler allied bases was on a riboflavin-containing system would receive some support

The starting material for this investigation was 3 4-xylidine, for gifts of which we are indebted to Imperial Chemical Industries Ltd , and Roche Products Ltd. This base was converted by the action of chloralhydrate and hydroxylamine hydrochloride by Sandmeyer's process (1919) into *iso*nitrosoacet-3 : 4-xylidide (VI), a by-product being *NN'-bis*-(3 . 4-xylyl)-urea On treatment of (VI) by Sandmeyer's process with sulphuric acid, four substances were formed, 5·6-dimethyl*is*atin (VII), 4 · 5-*dimethylisatin* (VIII), 6-*sulphoisonitrosoacet-3 4-xylidide* (IX) and its cyclization product 4 5-*dimethyl-7-sulphoisatin* (X). If the constitution of 5 . 6-dimethyl*is*atin (VII), which was known previously, is accepted, then the isomeric dimethyl*is*atin, formed simultaneously, must be (VIII). This structure was proved by an independent method as follows. On acetylation it gave *N-acetyl*-4 : 5-

dimethylisatin, which, on boiling with alkali (Camps 1899), yielded 2-*hydroxy*-5·6-*dimethylquinoline-4-carboxylic acid*. The hydroxyl group was replaced by chlorine and then by hydrogen, and the 5.6-*dimethylquinoline-4-carboxylic acid* so produced on decarboxylation gave 5:6-dimethylquinoline. The melting-point of this base and of its picrate agreed substantially with those attributed to these substances by Manske, Marion & Leger (1942). In a similar manner 5:6-dimethylisatin, which was the main product·of the Sandmeyer process, was converted into 6:7-dimethyl-quinoline-4-carboxylic acid, and a sample of this on decarboxylation gave 6:7-dimethylquinoline. The melting-point of the base and of the picrate agreed substantially with Manske's figures.

(VI)   (VII)

(VIII)   (IX)   (X)
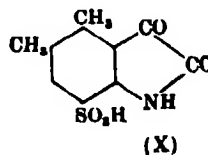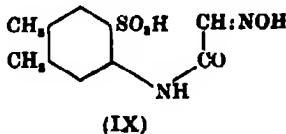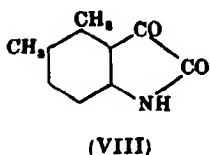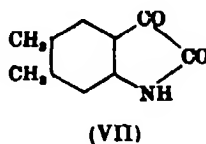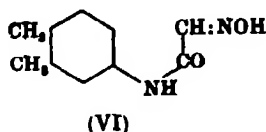
The constitution of the two sulphonic acids (IX) and (X) follows by a more indirect route On sulphonation of *iso*nitrosoacet-3 4-xylidide, the most probable position for the entering sulphonic acid group is the 6-position (IX). Such a 6-sulpho*iso*-nitrosoacet-3 4-xylidide on cyclization could only give one isatin which should be 4 5-dimethyl-5-sulphoisatin (X), and experimentally only one isatin was found On condensation with acetophenone and alkali by Pfitzinger's process (1897) it gave 2-*phenyl*-5.6-*dimethyl-8-sulphoquinoline-4-carboxylic acid* (XI), and on replacement of the sulpho-group by bromine by the method of Fieser & Bowen (1940) it yielded 2-*phenyl*-5.6-*dimethyl-8-bromoquinoline-4-carboxylic acid* (XII) On catalytic reduction this acid afforded 2-*phenyl*-5:6-*dimethylquinoline-4-carboxylic acid* (XIII), the constitution of which follows from its direct synthesis by a Pfitzinger reaction on 4:5-dimethylisatin. It was quite distinct from 2-phenyl-6 7-dimethylquinoline-4-carboxylic acid (XIV, $R = Ph$), which was prepared by a Pfitzinger reaction on 5 6-dimethylisatin. If the sulpho-group had been in the 2-position in *iso*nitrosoacet-3.4-xylidide, the final acid should have been 2-phenyl-6:7-dimethylquinoline-4-carboxylic acid. The 5-position for the sulpho-group in *iso*nitrosoacet-3·4-xylidide is unlikely on general grounds, and such a substance on cyclization should yield two isomeric sulphodimethylisatins, whereas only one was observed.

(XI)   (XII)   (XIII)   (XIV)
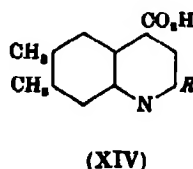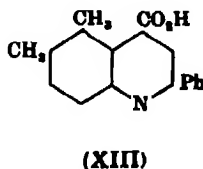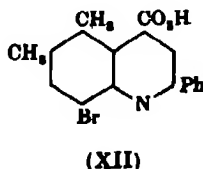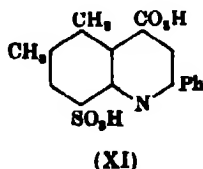
From 5:6-dimethylisatin, two cinchoninic acids were thus available in quantity, namely, 6:7-dimethylcinchoninic acid (XIV, $R = H$) and 2-phenyl-6:7-dimethyl-cinchoninic acid (XIV, $R = Ph$), for conversion into a series of parallel carbinol-amines allied to (III). 6:7-Dimethylcinchoninic acid (XIV, $R = H$) furnished an *ethyl* ester which on condensation with ethyl acetate in the presence of sodamide gave *ethyl 6:7-dimethyl-4-quinoloylacetate* (XV, $R = H$) mixed with *6:7-dimethyl-cinchoninamide*. On gentle acid hydrolysis the former furnished *6:7-dimethyl-4-acetoquinoline* (XVI, $R = H$), which on bromination in warm acetic acid gave *6:7-dimethyl-4-bromoacetoquinoline* (XVII, $R = H$). The latter was also obtained by bromination of ethyl 6:7-dimethylquinoloylacetate (XV, $R = H$).

(XV)   (XVI)

(XVII)   (XVIII)
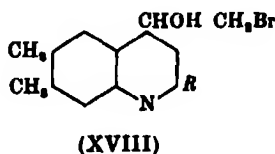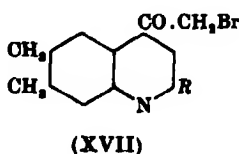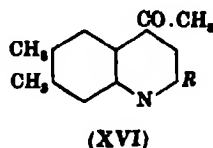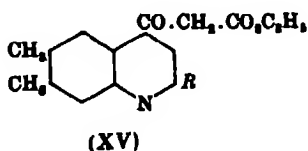
By a precisely similar series of reactions *2-phenyl-6·7-dimethyl-4-bromoaceto-quinoline* (XVII, $R = Ph$) was obtained from 2-phenyl-6·7-dimethylcinchoninic acid (XIV, $R = Ph$). These two ketobromides readily reacted with morpholine and piperidine to give a series of keto-bases which were catalytically reduced to carbinol-amines under the conditions used by King & Work (1940), a by-product formed by some mechanism which is obscure being 2-phenyl-6·7-dimethylcinchoninic acid (XIV, $R = Ph$) in the case of the 2-phenyl bases When, however, 6:7-dimethyl-4-bromoacetoquinoline (XVI, $R = H$) was allowed to react with *n*-dibutylamine, *n*-diamylamine or *n*-dihexylamine and the reaction product reduced catalytically, only methyl-6:7-dimethylquinolyl-4-carbinol (XXII) could be isolated. To avoid this fission of the ketoamines a device was used which was kindly communicated to us by Dr Lyndon F Small, of the National Institute of Health, Washington.

The ketobromides (XVII, $R = H$ or Ph) were reduced with aluminium *iso*-propylate by Ponndorf's method (1926) to the *carbinol-bromides* (XVIII, $R = H$ or Ph) which were relatively stable crystalline substances when isolated as their *hydrobromides*. When the latter were allowed to react in boiling benzene solution with an excess of dibutylamine, diamylamine or dihexylamine, they readily gave the six required *carbinolamine dihydrobromides* (XIX, $R = H$ or Ph, $R' = n\text{-}C_4H_9$, $n\text{-}C_5H_{11}$, $n\text{-}C_6H_{13}$)

The case of *morpholinomethyl-2-phenyl-6·7-dimethyl-4-quinolylcarbinol dihydro-bromide* (XX) is of special significance. This substance was prepared either by the action of morpholine on the ketobromide followed by catalytic reduction or by the action of morpholine on the corresponding carbinolbromide. The identity of the

product by the two processes indicates that in the action of amines on the carbinol-bromides where an intermediate ethyleneoxide is possible, the amine takes the terminal position. Lutz, Bailey, Clark, Codington, Deinet, Freek, Harnest, Leake, Martin, Rowlett, Salsbury, Shearer, Smith & Wilson (1946) and other American workers in this field also came to the same conclusion.

(XIX)   (XX)

(XXI)
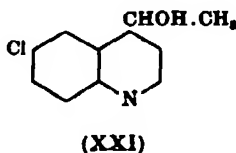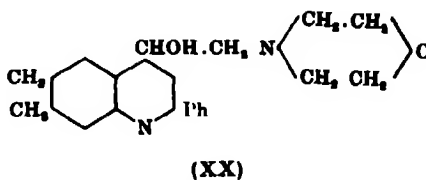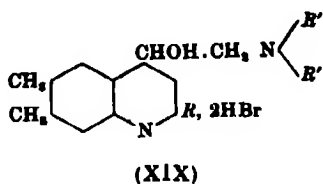
Although the use of the Ponndorf reaction on the ketobromides mentioned above gave the required carbinolbromides, when applied to 6-chloro-4-bromoaceto-quinoline with prolonged boiling the product of reduction was *methyl-6-chloro-quinolyl-4-carbinol* (XXI).

The ten carbinolamines described in this communication were tested for anti-plasmodial activity on *Plasmodium relictum* in canaries Our grateful thanks are due to Dr Ann Bishop of the Molteno Institute, Cambridge, for the results shown in table 1.

A perusal of this table shows that no piperidinomethyl or morpholinomethyl derivative in either series was active. The dibutylaminomethyl, diamylaminomethyl and dihexylaminomethyl derivatives, H, I and J in the 2-phenylquinoline series, were markedly active, having chemotherapeutic indices of $\frac{1}{4}$, $\frac{1}{3}$ and $\frac{1}{4}$ respectively. In the series without the 2-phenyl groups the dibutylaminomethyl derivative, C, showed no activity, whilst the diamylaminomethyl and dihexylaminomethyl derivatives, D and E, showed definite activity.

It is now of importance to compare the activity of the above-mentioned active bases with their 6-methoxyquinolyl analogues. King & Work (1940) have recorded the activities of the dibutylaminomethyl-, diamylaminomethyl- and dihexyl-aminomethyl-6-methoxy-4-quinolylcarbinols as determined by Miss Bishop on *P. relictum* in canaries by her standard technique. All three bases were active, but apart from the inactivity of dibutylaminomethyl-6 7-dimethyl-4-quinolylcarbinol C, and the slight activity of the dibutylaminomethyl-6-methoxy-4-quinolyl-carbinol, there is nothing to choose between the activities of the two series of bases. The replacement therefore of the 6-methoxy group by two methyl groups in the 6 : 7-positions corresponding to those in riboflavin has not led to any eutherapeutic effect, and no support is forthcoming for the view that a part at least of the anti-plasmodial action of quinine is due to a riboflavin antagonism.

TABLE 1

substance*	dose in mg./20 g. body wt.	day of appearance of parasites in blood	remarks
A. Piperidinomethyl- 6·7-dimethyl-4-quinolylcarbinol	6 × 5† 6 × 5 5 × (2 × 5) + 5	5 5 7	M.T.D.
B. Morpholinomethyl- 6:7-dimethyl-4-quinolylcarbinol	6 × 2·5 6 × 2·5 5 × (2 × 2·5) + 2·5	5 7 7	M.T.D.
C. Dibutylaminomethyl- 6.7-dimethyl-4-quinolylcarbinol	6 × 1·25 6 × 1 25	7 7	M.T.D.
D. Diamylaminomethyl- 6:7-dimethyl-4-quinolylcarbinol	6 × 2·5 6 × 2·5	12 5	M.T.D.
E. Dihexylaminomethyl- 6:7-dimethyl-4-quinolylcarbinol	4 × 10 + 2 × 5 2 × 10 + 4 × 5 1 × 10 + 3(2 × 10) + (2 × 5) + 5‡	14 9 16	M.T.D.
F. Piperidinomethyl-2-phenyl- 6.7-dimethyl-4-quinolylcarbinol	6 × 1·25 6 × 1·25	7 8	M.T.D.
G. Morpholinomethyl-2-phenyl- 6.7-dimethyl-4-quinolylcarbinol	6 × 2·5 6 × 2·5	7 7	M.T.D.
H. Dibutylaminomethyl-2-phenyl- 6 7-dimethyl-4-quinolylcarbinol	6 × 10 6 × 10 6 × 5 6 × 5 6 × 2·5 6 × 2·5 6 × 1·25 6 × 1·25	sterile 19 30 sterile 30 21 9 7	M.T.D. C/T = ½
I. Diamylaminomethyl-2-phenyl- 6.7-dimethyl-4-quinolylcarbinol	6 × 10 6 × 10 6 × 5 6 × 5 6 × 2 5 6 × 2 5 6 × 1·25 6 × 1·25	sterile sterile sterile sterile 16 12 9 14	M.T.D. C/T = ⅜
J. Dihexylaminomethyl-2-phenyl- 6.7-dimethyl-4-quinolylcarbinol	6 × 10 6 × 10 6 × 5 6 × 5 6 × 2·5 6 × 2·5 6 × 1·25 6 × 1 25	sterile sterile 23 23 16 30 10 9	M.T.D. C/T = ½
Control birds	—	5 to 7	—

* All as dihydrobromides.

† A dose of 5 mg. was given daily for 6 days

‡ A dose of 10 mg. on the first day, two doses each of 10 mg. on the next 3 days, 2 doses of 5 mg. on the 5th day and one of 5 mg. on the 6th day.

The three 6-methoxyquinolyl bases in the 2-phenyl series corresponding to H, I and J were not available for direct comparison on *P. relictum* in canaries In *A survey of antimalarial drugs* (Wiselogle 1946), however, the results are recorded of the anti-plasmodial tests of these three substances against *P. lophurae* in ducks and *P. gallinaceum* in chickens Their quinine equivalents were about unity, that is, on the same dose as quinine they produced the same lowering of parasitemia. Their dimethyl counterparts, the base H, I and J, were more active than quinine on *P. relictum* in canaries, but it is not possible to evaluate any specific contribution of the two methyl groups in the 6·7-positions from a comparison of tests on different parasites in different hosts.

During the course of this work the opportunity has been. taken of preparing the methochlorides of 6-*chloro-4-cinchoninamide*, 6.7-*dimethyl-4-cinchoninamide*, 2-*phenyl-6:7-dimethyl-4-cinchoninamide*, 2-phenyl-6:7-dimethylquinoline and 2-phenyl-5.6-dimethylquinoline, and to examine them for amoebicidal action on *Entamoeba histolytica in vitro.* Our thanks are due to Dr J. D. Fulton of this Institute for the tests They were either inactive or of a very low order of activity. When an attempt was made to prepare the quaternary methosulphate from methyl-5.6-dimethylquinolyl-4-carbinol (XXII) the product was found to be *β-(N:5.6-trimethylquinolyl)-ethylsulphuric acid betaine* (XXIII).

(XXII)　　　　　　(XXIII)
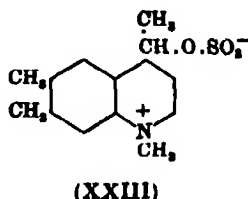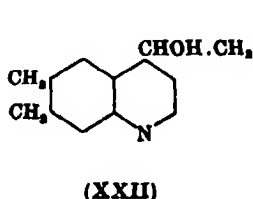
EXPERIMENTAL

Preparation of isonitrosoacet-3:4-*xylidide* (VI) (cf Marvel & Hiers 1932; Sandmeyer 1919) To a solution of chloral hydrate (45 g.) in water (600 c c) the following were added. crystalline sodium sulphate (650 g), a solution of 3·4-dimethylaniline (30·25 g.) in hydrochloric acid (150 c.c. water, 21·5 c.c concentrated hydrochloric acid), and a solution of hydroxylamine hydrochloride (55 g) in water (250 c c.). The mixture was heated rapidly until boiling began, and then for a further 2 min. The flask was cooled in running water and the solid collected.

The crude solid from four preparations was dissolved in the minimum quantity of cold potassium hydroxide (2N) and filtered to remove traces of a by-product (see below). Sodium chloride (100 g.) was added, causing the separation of the sparingly soluble *sodium* salt. This was collected, dried in air and recrystallized from spirit, filtering while hot to remove inorganic salts, it separated in colourless needles (Found: Na, 10·9. $C_{10}H_{11}O_2N_2Na$ requires Na, 10·8 %) A further crop was obtained by evaporating the alcoholic mother liquors. The residue on removing the alcohol was mixed with the aqueous filtrate of the original sodium salt and acidified with strong hydrochloric acid. The crude iso*nitrosoacet-3·4-xylidide* which was precipitated was

purified through the sodium salt as above (total yield 138 g., 72 %). The pure acid was prepared by the addition of glacial acetic acid to an alcoholic solution of the sodium salt; it crystallized in tiny colourless needles, m p. 179° C (Found: C, 62·5; H, 6·3; N, 14·6. $C_{10}H_{12}O_2N_2$ requires C, 62·5; H, 6·3; N, 14·6 %). It was, however, found advantageous to use the sodium salt instead of the free base for the cyclization, since it is more stable, more readily dried and more easily purified.

The effect of heating the alkaline solution was to cause the formation of large amounts of the by-product. This was shown to be $N:N'$-di-(3:4-dimethyl-phenyl) urea. It crystallized from glacial acetic acid in long colourless prisms, m p. 236° C (Cazeneuve & Moreau (1897) give m.p 234 to 235° C) (Found: C, 76·0; H, 7·5; N, 10·4. Calc. for $C_{17}H_{20}ON_2$· C, 76·1, H, 7·5, N, 10·4 %). It has a sweet taste. On the gradual addition of this urea (1 g.) to a cold solution of nitric acid (5 c.c. D 1·5) in glacial acetic acid (15 c c) there was a transient red coloration and N N'-di-(6-nitro-3 : 4-dimethylphenyl) urea separated in yellow needles, m.p. 270° C, in good yield. It was recrystallized from pyridine from which it separated in pale yellow prisms, m.p. unchanged (Found· C, 56·6; H, 5·1, N, 15·4 $C_{17}H_{18}O_5N_4$ requires C, 57·0; H, 5·0, N, 15·6 %).

5 : 6-*Dimethylisatin* (VII). This compound has been obtained previously by Kränzlein (1937) by the oxidation of the corresponding indigo

The dry sodium derivative of 3 4-dimethyl-*iso*-nitrosoacetanilide (107 g.) was added gradually to concentrated sulphuric acid (500 c.c.), which was stirred mechanially and cooled in an ice-salt mixture The solution was at first orange, but later became deep purple. When solution of the solid was complete, the temperature was raised and maintained at 75° C for 40 min., the solution cooled and poured on to crushed ice (1·5 kg). The solid which separated was at first orange but gradually became bright red. Next day it was collected and without washing (since this partially removed the main by-products) dissolved in the *minimum* quantity of strong sodium hydroxide solution (50 %). A deep purple solution was obtained which was heated to boiling and acidified strongly with concentrated hydrochloric acid. The excess of acid and the action of heat accelerated the ring closure of the yellow isatinic acid to give the brilliant red isatin. Next day the solid was collected, washed with water and dried at 85°; it was then separated into its two main components A (soluble) and B (insoluble) by extraction with boiling spirit (400, 200 and 100 c.c.). The combined filtrates containing A were evaporated until crystallization began, the required isatin separating in brilliant red needles or prisms, m.p. 209 to 213° C (37 g., 42 %). The melting-point could be raised to 215° (lit. 214 to 215° C) by two further crystallizations from the same solvent, but the once-crystallized product was found to be sufficiently pure for use in subsequent reactions. A further small crop (3 g., m p. 204 to 208 °C) was obtained by evaporation of the mother liquors. Attempts to isolate the isomeric 4 : 5-dimethylisatin at this stage were unsuccessful. The two isomeric isatins are more readily obtained pure through their acetyl derivatives.

N-Acetyl-5 . 6-*dimethylisatin* and *N-acetyl*-4 · 5-*dimethylisatin*. The crude 5 : 6-dimethylisatin, m.p. 209 to 213° C obtained in the previous stage was acetylated by the method of Camps (1899), except that longer heating was found to be necessary. The isatin (17·5 g.) was refluxed gently for 2 hr. with acetic anhydride (35 g.). On

cooling the *N-acetyl-5 : 6-dimethylisatin* separated in long rectangular yellow prisms or needles contaminated with a purple oil. The solid was collected, washed with a little benzene and recrystallized from the same solvent (230 c.c.); it separated in yellow prisms, m.p. 182° C (17 g., 80 %) (Found: C, 66·5; H, 5·1, N, 6·5. $C_{12}H_{11}O_3N$ requires C, 66·4, H, 5·1, N, 6·5 %). If crystallized several times from alcohol it was converted into *ethyl N-acetyl-4 . 5-dimethylisatinate*, very pale yellow prisms, m.p. 103° C (Found· C, 63·9; H, 6·5; N, 5·4. $C_{14}H_{17}O_4N$ requires C, 63·9, H, 6·5; N, 5·3 %). The corresponding free acid, *N-acetyl-4 : 5-dimethylisatinic acid*, was also isolated in small amounts. It crystallized in pale yellow prisms from alcohol, m.p 196° C (Found: C, 61·3; H, 5·9, N, 6·3. $C_{12}H_{13}O_4N$ requires C, 61·3; H, 5·6, N, 6·0 %). Both the free acid and the ester reacted in the same way as *N*-acetyl-5 . 6-dimethylisatin in the subsequent treatment with a boiling solution of sodium hydroxide.

N-Acetyl-5 : 6-dimethylisatin on hydrolysis with boiling 16 % hydrochloric acid gives 5 : 6-dimethylisatin (VII), m p. 215° C, and this is the best method for obtaining this isatin pure. *N-Acetyl-5 : 4-dimethylisatin* was isolated from the acetic anhydride benzene filtrates by fractional crystallization from benzene-ligroin mixtures. It crystallized from benzene-*cyclo*hexane mixtures in glistening golden plates, m.p. 128° C (Found: C, 65·9, H, 5·1, N, 6·8. $C_{12}H_{11}O_3N$ requires C, 66·4, H, 5·1, N, 6·5 %). It was considerably more soluble than its isomeride in all solvents (1 g. of *N*-acetyl-5 . 6-dimethylisatin dissolved in 12 c.c. of boiling benzene, but only 2 c.c. were required for the same weight of the isomeride). Yield of the isomeride isolated was 1·5 g. (7 %), but probably considerably more was formed. A mixed melting-point of the isomerides showed a definite depression (114° C). *4 5-Dimethylisatin* (VIII) was prepared from its acetyl derivative by refluxing for 30 min. with hydrochloric acid (16 %). After several crystallizations from methyl ethyl ketone it was obtained in the form of square red plates, m p. 222° C (Found. C, 68·4, H, 5·2, N, 8·3 $C_{10}H_9O_2N$ requires C, 68·6, H, 5·1, N, 8·0 %) A mixed melting-point with the isomeric dimethylisatin showed a marked depression (185° C).

SULPHONATED BY-PRODUCTS

The solid, B, remaining undissolved after the alcoholic extraction, was shown to be *sodium 4 : 5-dimethylisatin-7-sulphonate* It crystallized from water in orange needles, unmelted at 300° C. (Found C, 42·9, H, 3·0, N, 5·2, S, 11·2, Na, 8·9. $C_{10}H_8O_5NSNa$ requires C, 43·2; H, 2·9, N, 5·1, S, 11·6, Na, 8·3 %) (Yield, 34 g , 25 % of the theoretical). If the initial precipitate of the mixed isatins was washed with water most of the sulphonic acid was removed, but this was a less satisfactory method of separation than alcoholic extraction of the isatins after reaction with sodium hydroxide.

The free *4 : 5-dimethylisatin-7-sulphonic acid* (X) was best prepared through the barium salt; it crystallized from water in orange needles unmelted at 300° C (Found. C, 47·3; H, 4·3; N, 5·3. $C_{10}H_9O_5NS$ requires C, 47·1, H, 3·5; N, 5·5 %).

Small amounts of *6-sulpho-3 . 4-dimethylisonitrosoacetanilide* (IX) were also isolated by washing the original solid from the cyclization process with water before treatment with alkali. It crystallized from alcohol in yellow needles, unmelted at

300° C (Found: C, 44·2; H, 4·2; N, 10·4. C$_{10}$H$_{12}$O$_5$N$_2$S requires C, 44·1; H, 4·4; N, 10·3). It is cyclized by further treatment with sulphuric acid to the above isatinsulphonic acid.

2-*Phenyl-5 : 6-dimethyl-8-sulphoquinoline-4-carboxylic acid* (XI). Sodium 4:5-dimethylisatin-7-sulphonate (27·7 g.), acetophenone (20 c.c.) and a solution of sodium hydroxide (16 g.) in water (50 c.c.) were heated together on a steam bath with mechanical stirring for 16 hr.; at the end of this time all the solid had dissolved. The mixture was evaporated under reduced pressure to a small volume, and on cooling the *sodium* salt of 2-*phenyl-5 : 6-dimethyl-8-sulphoquinoline-4-carboxylic acid* separated as a purple oil which crystallized on rubbing with alcohol. It could be purified by crystallization from alcohol or water and was obtained in colourless needles, unmelted at 300° C (Found: Na, 6·3. C$_{18}$H$_{14}$O$_5$NSNa requires Na, 6·1 %). The free *di-acid* was not precipitated from the solution of the mono-sodium salt by the addition of acetic acid, but on acidification with mineral acids it separated in tiny colourless needles, m.p. 298° (Found C, 60·2; H, 4·4; N, 4·0. C$_{18}$H$_{15}$O$_5$NS requires C, 60·5, H, 4·2; N, 3·9 %). It was very sparingly soluble in water, alcohol and acetic acid and very soluble in pyridine

8-*Bromo-4-carboxy-2-phenyl-5·6-dimethylquinoline* (XII) (cf Fieser & Bowen 1940). To a solution of 4-carboxy-2-phenyl-5.6-dimethylquinolyl-8-sulphonic acid (7·14 g) in potassium hydroxide solution (400 c.c 0·2 N) at 70° C a solution of bromine (1·6 c.c.) in potassium bromide solution (8 g. in 40 c.c. of water) was added. An oil separated and the bromine was absorbed at first immediately, then slowly, when the addition was complete, the solution was acidic and contained a small amount of free bromine. The temperature was maintained at 70 to 75° C for 1 hr. On cooling, the oil solidified, it was collected and dried *in vacuo* (9·2 g , m.p. 110° C) Extraction of the solid with boiling alcohol left a small residue of insoluble material which was shown to be 2-*phenyl-5·6-dimethylquinoline-8-sulphonic* acid (XIII) unmelted at 350° C (Found· C, 64·8, H, 4·5, N, 4·8 C$_{17}$H$_{15}$O$_3$NS requires C, 65 2, H, 4·8; N, 4·5 %). It was sparingly soluble in all the usual solvents but could be purified by crystallization of its *sodium salt* from alcohol; this separated in beautiful long colourless needles, unmelted at 350° C (Found Na, 6·8 C$_{17}$H$_{14}$O$_3$NSNa requires Na, 6·9 %). Evaporation of the alcoholic solution gave a crystalline solid which was extracted with hot potassium hydroxide solution, a small amount of 8-*bromo-2-phenyl-5.6-dimethylquinoline* remaining undissolved. After several crystallizations from alcohol this was obtained in slightly yellow prisms, m p. 183° C (Found: C, 64·9, H, 4·7, N, 4·4. C$_{17}$H$_{14}$NBr requires C, 65·4, H, 4·5, N, 4·5 %).

The main product, 2-*phenyl-5·6-dimethyl-8-bromoquinoline-4-carboxylic acid* (XII), was obtained from the alkaline solution by acidification with glacial acetic acid. It crystallized from benzene-alcohol mixtures in faintly yellow needles, m p 220° C (Found: C, 61·3, H, 4·1; N, 4·0. C$_{18}$H$_{14}$O$_2$NBr requires C, 60·7; H, 3·9; N, 3·9 %) It could also be purified by crystallization of the hydrobromide, beautiful yellow needles from acetone-hydrobromic acid mixtures, m.p. 148° C, or by means of the sparingly soluble potassium salt

2-*Phenyl-5 : 6-dimethylcinchoninic acid* (XIII). The above 2-phenyl-5 : 6-dimethyl-8-bromoquinoline-4-carboxylic acid (3·56 g.) was dissolved in N-potassium hydroxide

solution (50 c.c.) and hydrogenated for 24 hr. with a palladized charcoal catalyst. The catalyst was collected and the filtrate boiled and acidified to Congo; on cooling the 2-*phenyl*-5 : 6-*dimethylcinchoninic acid* (2·5 g.) crystallized in faintly yellow prisms, m.p. 264° C. It was purified by reprecipitation from sodium hydroxide solution with acetic acid and obtained in tiny colourless needles, m.p. 266° C (Found: C, 78·0, H, 5·5; N, 5·1. $C_{18}H_{15}O_2N$ requires C, 78 0; H, 5·4; N, 5·1 %). It was identical with the acid prepared by a Pfitzinger reaction on 4 : 5-dimethyl-isatin as described below Mixed melting-point determinations with 2-phenyl-6·7-dimethylcinchoninic acid showed marked depressions

2-*Phenyl*-5 · 6-*dimethylcinchoninic acid* was prepared from 4 · 5-dimethylisatin by the method of Kränzlein (1937), except that sodium hydroxide was used instead of potassium hydroxide. The product crystallized as the *sodium* salt from the reaction mixture and this could be recrystallized from alcohol. It separated in beautiful long colourless needles, unmelted at 300° C (Found Na, 8·4 $C_{18}H_{14}O_2NNa$ requires Na, 8·0 %). 2-*Phenyl*-5 . 6-*dimethylcinchoninic acid* crystallized from a solution of the sodium salt in alcohol on the addition of glacial acetic acid in tiny colourless needles, m p 266° C (Found C, 78·1; H, 5·5, N, 5·1 $C_{18}H_{15}O_2N$ requires C, 78·0; H, 5·4; N, 5·1 %) A mixed melting-point determination with 2-phenyl-6.7-dimethylcinchoninic acid showed a marked depression (232 to 242° C)

2-*Phenyl*-5 : 6-*dimethylquinoline* 2-Phenyl-5 : 6-dimethylcinchoninic acid (5·54 g.) copper bronze (5·54 g) and quinoline (50 c c) were boiled together for 4 hr , during which time considerable darkening occurred. The copper bronze was collected, washed with alcohol and the filtrate evaporated, after removal of the quinoline the required *base* crystallized (4·6 g , 100 % of theory). It crystallized from *cyclo*hexane in colourless shimmering plates, m p. 110° C (Found. C, 88 0, H, 6·4, N, 6·3. $C_{17}H_{15}N$ requires C, 87·6, H, 6 4, N, 6·1 %).

2-*Phenyl*-6 7-*dimethylquinoline* was prepared in the same way from 2-phenyl-6 7-dimethylcinchoninic acid It crystallized from alcohol in colourless silky needles, m p 147° C (Found. C, 87·7, H, 6·2, N, 6·1. $C_{17}H_{15}N$ requires C, 87·6; H, 6·4; N, 6·1 %).

Carbinolamines derived from 6 . 7-dimethylcinchoninic
and 2-phenyl-6 . 7-dimethylcinchoninic acids

6 : 7-*Dimethylcarbostyril*-4-*carboxylic acid* (cf. Ainley & King 1938) *N*-Acetyl-5 · 6-dimethylisatin (21·7 g) was refluxed for 1 hr. with sodium hydroxide (10 g) in water (600 c.c). The mixture was then acidified with concentrated hydrochloric acid and refluxed for a few minutes to convert isatinic acid to isatin. Next day the solid was collected and ground with saturated sodium bicarbonate solution which left the dimethylisatin (4·3 g.) undissolved. The filtrate was acidified and gave 6 : 7-*dimethylcarbostyril*-4-*carboxylic acid*, yield 15 g. (98 % allowing for recovered isatin), m p. 342° C. It could be recrystallized from glacial acetic acid from which it separated in fine colourless needles, unmelted at 350° C, although considerable darkening occurs at lower temperatures (Found· C, 66·1; H, 5·2; N, 6·8. $C_{12}H_{11}O_3N$ requires C, 66·4; H, 5·1; N, 6·5 %)

2-Chloro-6 : 7-dimethylcinchoninic acid was prepared by the method of Ainley & King (1938) using phosphorus oxychloride, except that owing to the sparing solubility of the sodium salt of the product, sodium bicarbonate could not be used for purification The acid was dissolved in a slight excess of potassium hydroxide solution (2 N) and the solution treated with charcoal and filtered through kieselguhr. Excess sodium chloride was added and the *sodium salt*, which separated slowly in beautiful long colourless needles, unmelted at 300° C (Found: Na, 8·7. $C_{12}H_9O_2NClNa$ requires Na, 8·9 %) was collected, dissolved in alcohol and precipitated with acetic acid. Evaporation of the alcoholic and acidification of the aqueous filtrates gave a small crop of crude acid, which was purified in the same way, yield 93 %. The pure *2-chloro-6 . 7-dimethylcinchoninic acid* crystallized from acetic acid in colourless needles, m p. 218 to 219° C, giving a cloudy liquid which cleared gradually at considerably higher temperatures (Found· C, 61·1; H, 4·3; N, 5·9. $C_{12}H_{10}O_2NCl$ requires C, 61·1; H, 4·2; N, 5·9 %).

6 . 7-Dimethylcinchoninic acid was also prepared by application of the method of Ainley & King (1938) in 95 % yield. The product crystallized from pyridine water mixtures in colourless needles, m.p 321° C (decomp.) (Found: C, 71·8; H, 5·6, N, 7·4. $C_{13}H_{11}O_2N$ requires C, 71·6, H, 5·5, N, 7·0 %).

Ethyl 6 : 7-dimethylcinchoninate was prepared from the acid by the action of thionyl chloride followed by alcohol. The yield was almost quantitative. The *ethyl 6 : 7-dimethylcinchoninate* crystallized from ligroin in large colourless prisms, m p. 68° C (Found: C, 73·6, H, 6·5, N, 6·2. $C_{14}H_{15}O_2N$ requires C, 63·4; H, 6·6, N, 6·1 %) It could also be purified by distillation, b.p. 168° C/3·0 mm.

Ethyl 6 : 7-dimethyl-4-quinoloylacetate (XVI, R = H) (cf. King & Work 1940). To a mixture of ethyl 6 : 7-dimethylcinchoninate (45·8 g.) and ethyl acetate (20 g.) dissolved in dry benzene (500 c c), finely divided sodamide (10 g.) was added and the mixture refluxed gently for 16 hr. and then poured into water. Next day the solid which had separated, which proved to be *6 · 7-dimethylcinchoninamide*, was collected (7·1 g.). It crystallized from alcohol in colourless needles, m p. 241° C (Found: C, 71·8; H, 6·1; N, 14·0. $C_{13}H_{12}ON_2$ requires C, 72·0, H, 6·0; N, 14·0 %). The aqueous layer was separated and extracted with benzene; the combined benzene solutions were evaporated giving unchanged ethyl 6 · 7-dimethylcinchoninate (8·5 g., m.p. 64° C). The aqueous layer was then saturated with carbon dioxide, causing the separation of *ethyl 6 : 7-dimethyl-4-quinoloylacetate* as an oil. It was best purified for analysis through the *hydrobromide*, which could be crystallized from methyl alcohol-ethereal hydrogen bromide mixture in pale yellow prisms, m.p 162° C (efferv.) (Found: C, 54·1; H, 5·2; N, 4·3. $C_{16}H_{17}O_3N$, HBr requires C, 54·5; H, 5·1; N, 4·0 %). The oil was separated and the aqueous layer extracted with ether, the ether evaporated and the residue combined with the oil; on standing it solidified, m p. 48° C. From the aqueous layer 6 : 7-dimethylcinchoninic acid (0·8 g.) was precipitated with concentrated hydrochloric acid.

The crude ethyl 6 : 7-dimethyl-4-quinoloylacetate (27 g.) was ground with hydrobromic acid (40 c.c. 24 %); 6 hr. later the hydrobromide was collected, washed with a small amount of the same acid and dried in a desiccator. (The yield was 27·3 g. of substance of indefinite melting-point, but it was found to be sufficiently pure

for use in subsequent reactions; this is a 58 % yield allowing for recovered ester, amide and acid.)

6 : 7-*Dimethyl-4-quinolyl bromomethyl ketone* (XVII, R = H) (cf. Rabe, Pasternack & Kindler 1917). Ethyl 6 : 7-dimethyl-4-quinoloylacetate hydrobromide (3·52 g.) was finely ground under hydrobromic acid (15 c c., 24 %), it was then treated with a solution of bromine (0·53 c.c.) in hydrobromic acid (5 c.c., 24 %) This caused the separation of a thick oil which dissolved on heating to 70° C. The temperature was maintained at 70 to 80° C for 1 hr., during which time carbon dioxide was evolved and some crystallization occurred. Finally, the temperature was raised to 85° C for 20 min. On cooling 6 : 7-*dimethyl-4-quinolyl bromomethyl ketone hydrobromide* crystallized in pale yellow needles, m p 218° C. It could be recrystallized from alcohol containing a few drops of 24 % hydrobromic acid or preferably from glacial acetic acid, from which it separated in buff-coloured prisms, m p. 236 to 238° C (Found: C, 44·0, H, 4·0, N, 4·0. $C_{13}H_{12}ONBr$, HBr requires C, 43·4, H, 3 6, N, 3·9 %).

Piperidinomethyl-6 . 7-dimethyl-4-quinolylcarbinol (cf. King & Work 1940). Powdered 6 . 7-dimethyl-4-quinolyl bromomethyl ketone hydrobromide (3·45 g.) was added gradually to a solution of piperidine (3 c c) in dry ether (50 c c) with continual shaking. After 1 hr. at room temperature the piperidine hydrobromide (2·25 g., 92 % of theoretical) was collected and the ether removed under reduced pressure without heating. The residue was dissolved in methyl alcohol (50 c c.) and hydrochloric acid (25 c.c. 3 N), both of which had been cooled to −5° C and hydrogenated using Adams's catalyst (0·1 g). Hydrogen absorption was completed in 8 hr. The catalyst was collected and the mixture evaporated at 50° C under reduced pressure, excess hydrogen chloride being removed by adding small amounts of water and evaporating several times. The reaction mixture was dissolved in water (50 c.c.) and extracted with chloroform which failed to extract any significant quantity of material. The solution was made alkaline with strong sodium hydroxide solution (50 %) and extracted with chloroform. The chloroform solution was evaporated and the residue (2·8 g.), which consisted of the required base contaminated with piperidine, was purified by crystallization of its *hydrobromide* from aqueous alcoholic hydrobromic acid, it separated in colourless needles, m p. 220° C (Found on air-dry solid; loss at 85° C, 6·4. $C_{18}H_{24}ON_2$, 2HBr, 2H_2O required H_2O, 6·6 %. On dried material. C, 48·3, H, 6·2; N, 6·2 $C_{18}H_{24}ON_2$, 2HBr requires C, 48·4, H, 5·8; N, 6·3 %). The *base* crystallized from *cyclo*hexane in colourless prisms, m.p. 132° C (Found: N, 9·7. $C_{18}H_{24}ON_2$ requires N, 9·9 %).

Morpholinomethyl-6 . 7-dimethyl-4-quinolylcarbinol was prepared in the same way as the piperidine analogue except, that since the reaction product was sparingly soluble in ether, the morpholine hydrobromide could not be removed from the reaction mixture before hydrogenation. The *dihydrobromide* of the product crystallized from water in colourless prisms, m.p. 200° C, yield 2·6 g. (Found. C, 44·3; H, 6·1, N, 6·0, H_2O, 4·5. $C_{17}H_{22}O_2N_2$, 2HBr, H_2O requires C, 43·8; H, 5·6; N, 6·0; H_2O, 3·9 %). The *base* crystallized from ethyl acetate in prisms, m.p. 164° C (Found· C, 71·2, H, 7·4; N, 9·9. $C_{17}H_{22}O_2N_2$ requires C, 71·3, H, 7·7; N, 9·8 %).

Using the same procedure attempts were made to prepare the carbinolamines containing the di-*n*-butyl, di-*n*-amyl and di-*n*-hexyl groups. In each case the only

product isolated was 6 : 7-*dimethyl-4-quinolylmethylcarbinol*, which was purified, in good yield, by crystallization of the *hydrobromide* from water; it separated in colourless needles, m.p. 228 to 229° C (Found: C, 55·3; H, 5·7; N, 5·0. $C_{12}H_{15}ON, HBr$ requires C, 55·3; H, 5·7; N, 5·1 %). The *base* crystallized from ethyl acetate in colourless prisms, m.p. 152° C (Found: C, 77·2, H, 7·0, N, 7·0. $C_{12}H_{15}ON$ requires C, 77·6, H, 7·4; N, 7·0 %).

On heating the base (1·3 g.) with dimethyl sulphate (3 c.c.) in benzene (25 c.c.) for 10 hr. and evaporation of the benzene, a substance was obtained (0·8 g) which crystallized from water in clusters of tiny, slightly yellow needles, m.p 270° C. It did not react with sodium iodide or barium chloride in aqueous solution, and analysis showed it to have the formula $C_{14}H_{17}O_4NS$ (Found: C, 56·8; H, 5·8, N, 4·9. $C_{14}H_{17}O_4NS$ requires C, 56·9, H, 5·8, N, 4·7 %). This can only be reconciled with the constitution β-(*N* : 5 : 6-*trimethylquinolyl*)-*ethylsulphuric acid betaine* (XXIII).

6 : 7-*Dimethyl-4-quinolyl methyl ketone* (XVI, $R = H$) Ethyl 6 . 7-dimethyl-4-quinoloylacetate (2·71 g) was refluxed for 3 hr. with 15 % sulphuric acid (50 c.c.), evolution of carbon dioxide proceeding rapidly during the first half hour. The solution was made alkaline, causing the precipitation of 6 . 7-*dimethyl-4-quinolyl methyl ketone* (1·3 g.) as a crystalline buff-coloured solid. It was purified by sublimation in a high vacuum (bath temperature 140° C) and crystallized from ligroin, from which it separated in very faintly yellow long slender prisms, m.p. 93° C (Found. C, 78·0; H, 6·8; N, 7·1 $C_{13}H_{13}ON$ requires C, 78·4, H, 6·5, N, 7·0 %). Acidification of the alkaline mother liquors gave a very small precipitate of 6 · 7-dimethylcinchoninic acid, but when alkaline hydrolysis was used, as in the case of the analogous 2-phenyl compound, the amount of this acid formed was considerable.

6 : 7-*Dimethyl-4-quinolyl bromomethyl ketone hydrobromide*. The above ketone (1·99 g) was dissolved in purified glacial acetic acid (15 c c), and to the stirred solution (external bath 65 to 70° C) was added bromine (0·7 c.c) in glacial acetic acid (5 c c) over a period of 5 min. The bromine was absorbed almost immediately, and when the addition was complete the solution became cloudy, and soon after yellow crystals began to separate The external temperature was raised to 100° C, kept there for 5 min. and then the solution allowed to cool to 0° C overnight. The crystalline *bromomethyl ketone hydrobromide* (3·5 g.) m p. 220° C, was collected and dried *in vacuo*, it crystallized from glacial acetic acid in buff-coloured prisms, m p 236 to 238° C, identical with the compound obtained by bromination of the quinoloylacetate as previously described.

6 : 7-*Dimethyl-4-bromomethylquinolylcarbinol* (XVIII, $R = H$). The above bromomethyl ketone hydrobromide (3·59 g.) was boiled for 2 hr. with aluminium *iso*propylate (2·1 g.) and dry *iso*propyl alcohol (100 c.c.). When boiling began the mixture became deep purple, but faded slowly during the first hour, all the acetone had distilled after $1\frac{1}{2}$ hr. boiling. N-Hydrobromic acid (50 c c.) was then added and the *iso*propyl alcohol removed under reduced pressure. The crystalline solid, which proved to be the required *carbinol hydrobromide*, was collected, yield 3·1 g. (85 %), m p. 220° C. It crystallized from glacial acetic acid in colourless slender prisms, m.p. 222° C (Found: C, 43·2; H, 4·2, N, 4·1. $C_{13}H_{14}ONBr, HBr$ requires C, 43·1; H, 4·2; N, 3·9 %).

Dibutylaminomethyl-6 : 7-*dimethylquinolylcarbinol dihydrobromide* (XIX, *R* = H, *R′* = C₄H₉). The preceding carbinol hydrobromide (2·9 g., m.p. 220° C), benzene (30 c.c.) and di-*n*-butylamine (6·8 c.c.) were boiled on the water-bath for 16 hr., during which time the solution went from cherry red to black. An excess of dry ether was added, the precipitated dibutylamine hydrobromide collected (3·0 g) and the organic solvents removed from the filtrate. N-sodium hydroxide (12 c.c) was then added and the liberated bases extracted with chloroform. The chloroform was removed and the residual dibutylamine distilled *in vacuo* at 110° C. The residual base was treated with N-hydrobromic acid (10 c c) and extracted with ether to remove a non-crystallizable gum. The acid solution was evaporated somewhat, and the required *hydrobromide* was obtained by fractional crystallization. It crystallized from methyl ethyl ketone—methyl alcohol mixtures in colourless prisms, m.p 161° C (Found. loss at 90° C, 4·0, C, 49·9, H, 7·0, N, 5·7. C₂₁H₃₂ON₂, 2HBr, H₂O requires C, 49·6; H, 7·1, N, 5·5, H₂O, 3·7 %).

Diamylaminomethyl-6 7-*dimethylquinolylcarbinol hydrobromide* (XIX, *R* = H, *R′* = C₅H₁₁). In a similar way the parent bromomethylquinolylcarbinol hydrobromide (2·9 g) and *n*-diamylamine (8·2 c c) gave the required hydrobromide which was recrystallized several times from methyl alcohol-ethyl acetate mixtures from which it separated in colourless prisms, m.p. 184° C (0·8 g) An air-dried specimen lost no weight on heating at 85° C (Found. C, 52 9, H, 6·9, N, 5·7. C₂₃H₃₆ON₂, 2HBr requires C, 53·2, H, 7·3, N, 5 4 %).

Dihexylaminomethyl-6 . 7-*dimethylquinolylcarbinol hydrobromide* (XIX, *R* = H, *R′* = C₆H₁₃) The bromomethylquinolylcarbinol hydrobromide (6·37 g.), *n*-dihexylamine (21·4 c c.) and benzene (50 c c.) were heated to boiling for 24 hr. The required base, freed from dihexylamine by fractional distillation, was treated with N-hydrobromic acid (39 c c) and the solution evaporated under reduced pressure until crystallization began The *hydrobromide* was crystallized several times from alcohol-ethyl acetate mixtures, from which it separated in colourless long slender prisms, m p. 181° C (Found on air-dried solid, loss at 100° C, 2·7. C₂₅H₄₀ON₂, 2HBr, H₂O requires H₂O 3 1 %. On dried solid· C, 54·3; H, 8·0, N, 5·4 C₂₅H₄₀ON₂, 2HBr requires C, 54·9, H, 7·7, N, 5·1 %)

2-*Phenyl*-6 : 7-*dimethylcinchoninic acid* was prepared from 5 6-dimethylisatin and acetophenone as described by Kränzlein (1937). The product crystallized from pyridine-water mixtures in colourless needles, m p. 250° C (lit. 251·5° C) (Found: C, 78·2; H, 5·4, N, 5·2. Calc for C₁₈H₁₅O₂N C, 78·0, H, 5·4, N, 5·1 %) The *sodium* salt, which was sparingly soluble in water, crystallized from alcohol in colourless needles unmelted at 300° C (Found Na, 8·4. C₁₈H₁₄O₂NNa requires Na, 8·0 %).

Ethyl 2-phenyl-6 7-*dimethylcinchoninate* was prepared by refluxing 2-phenyl-6 7-dimethylcinchoninic acid (27·7 g) with thionyl chloride (100 c.c.) for 2 hr. The excess thionyl chloride was removed under reduced pressure and alcohol (100 c c) was added to the reaction mixture which was then refluxed for a further 2 hr. The alcohol was evaporated under reduced pressure until crystallization began. Next day the hydrochloride was collected, washed with a little acetone (yield 34 g.) and stirred with sodium hydroxide solution (100 c c 2N). *Ethyl 2-phenyl*-6 : 7-*dimethylcinchoninate* crystallized in prisms, m p. 95° C, yield 29 g. It was recrystallized from

alcohol from which it separated in colourless strongly refractive prisms, m.p. 101 to 102° C (Found: C, 78·7; H, 6·5; N, 5·0. $C_{20}H_{19}O_2N$ requires C, 78·7; H, 6·2; N, 4·6 %).

Ethyl 2-phenyl-6 : 7-dimethyl-4-quinoloylacetate (XV, $R = $ Ph) was prepared by the same method as 6 : 7-dimethyl-4-quinoloylacetate (described above), but the different solubilities of the products necessitated the use of a different method of separation.

The reaction mixture (from 30·5 g of ethyl 2-phenyl-6 : 7-dimethylcinchoninate, ethyl acetate and sodamide), several hours after pouring into water was filtered and the solid washed repeatedly with ether until the yellow oil had dissolved and only colourless crystalline material remained. This consisted of almost all the 2-*phenyl*-6 · 7-*dimethylcinchoninamide* which had been formed in the reaction and some *ethyl sodio-2-phenyl-6 7-dimethyl-4-quinoloylacetate*, the latter being moderately soluble in cold ether. The amide crystallized from glacial acetic acid in small colourless needles, m.p. 258° C (Found C, 78·3; H, 6·1; N, 10·1. $C_{18}H_{16}ON_2$ requires C, 78·3, H, 5·8; N, 10·1 %) (Yield 6·0 g , 24 %) The sodio-ester crystallized from ethyl acetate in faintly yellow needles, m p 132° C (Found: N, 3·6. $C_{22}H_{20}O_3NNa$ requires N, 3·8 %). The separation of the mixture into its components was best carried out by grinding the air-dried solid with excess dilute acetic acid (N/2), collecting, again air-drying and extracting with boiling benzene. Whereas the amide was only sparingly soluble in this solvent the free *ethyl 2-phenyl-6 : 7-dimethyl-4-quinoloylacetate* dissolved readily. The benzene was evaporated and the residual solid crystallized several times from alcohol, it separated in colourless needles, m p. 108° C (Found C, 76·1; H, 6·1, N, 4·0. $C_{22}H_{21}O_3N$ requires C, 76·1, H, 6·2; N, 4·2 %).

The ether washings and the benzene layer from the reaction mixture were evaporated and combined. They contained ethyl sodio-2-phenyl-6 . 7-dimethyl-4-quinoloylacetate mixed with unchanged ethyl 2-phenyl-6 . 7-dimethylcinchoninate, and these could be separated by fractional crystallization, but owing to their similar solubilities this method was wasteful, and it was found better to proceed to the next stage, hydrolysis with alkali, when the products ketone and acid could readily be separated. When a separation was carried out before hydrolysis 9·2 g. of original ester was recovered, and the yield of the required product was 10 4 g ; this is 68 % allowing for recovered ester and amide.

Saturation of the aqueous layer with carbon dioxide gave no significant precipitate, showing that the sodium derivative of the required ester is very sparingly soluble in water, but with hydrochloric acid a small amount (1·25 g.) of crude 2-phenyl-6 : 7-dimethylcinchoninic acid was obtained.

Recovery of 2-phenyl-6 : 7-dimethylcinchoninic acid from its amide. 2-Phenyl-6 . 7-dimethylcinchoninamide (55·2 g.) was refluxed with a mixture of concentrated hydrochloric acid (750 c.c.) and water (750 c.c.) for 24 hr. The colourless amide dissolved and a finely crystalline yellow hydrochloride separated. The reaction mixture was made alkaline with potassium hydroxide and filtered to remove unchanged amide (7 g.). On acidification of the filtrate with glacial acetic acid the required acid was obtained in good yield (47 g.).

2-Phenyl-6 : 7-dimethyl-4-quinolyl methyl ketone (XVI, $R = $ Ph). Ethyl 2-phenyl-6 :7 -dimethyl-4-quinoloylacetate (17·36 g.) was boiled for 2 hr. with 0·5 N-sodium

hydroxide. Ether extraction (500 c.c.) gave 2-*phenyl*-6 : 7-*dimethyl*-4-*quinolyl methyl ketone* (12·5 g.) which crystallized from absolute alcohol (75 c.c.) in prisms, m.p. 127 to 128° C (Found· C, 83·1; H, 6·4. $C_{19}H_{17}ON$ requires C, 82·9, H, 6·2 %).

2-*Phenyl*-6 : 7-*dimethyl*-4-*quinolyl bromomethyl ketone* (XVII, $R = $ Ph). The above ketone (2·75 g) was dissolved in purified glacial acetic acid (27·5 c c.) and to the stirred solution (external bath 60 to 65° C) was added bromine (0·53 c.c) in glacial acetic acid (5 c.c.) over a period of 5 min. The external temperature was then raised to 100° C, kept there for 5 min. and then the solution allowed to cool to 0° C overnight. The crystalline *bromomethyl ketone hydrobromide* (3·7 g.), m p. 218 to 220° C, was collected and dried *in vacuo* (Found: C, 52·2, H, 4·3; N, 3·4. $C_{19}H_{16}ONBr$, HBr requires C, 52·4; H, 3·9, N, 3·2 %).

2-*Phenyl*-6 . 7-*dimethyl*-4-*bromomethylquinolylcarbinol* (XVIII, $R = $ Ph) The preceding bromomethyl ketone hydrobromide (3·2 g) was boiled for 1·5 hr. with aluminium *iso*propylate (1·5 g) and dry *iso*propyl alcohol (90 c c). The intense indigo-blue colour formed on first boiling gradually faded as the reaction proceeded, all acetone having distilled after 1 hr. boiling. N-hydrobromic acid (50 c.c.) was then added and the *iso*propyl alcohol removed under reduced pressure The crystalline solid, which proved to be the required *carbinol hydrobromide*, was collected, yield 3·1 g., m.p. 218° C (efferv). A small specimen was crystallized from 100 vol. of boiling acetic acid and separated in minute needles, m p. 216° C (efferv.) It can also be conveniently crystallized from alcohol containing a few drops of 3N-aqueous hydrobromic acid (Found: C, 52·2; H, 4·6. $C_{19}H_{18}ONBr$, HBr requires C, 52·2; H, 4·4 %). Some preparations of this substance melt at 278° C (decomp.) and crystallize from acetic acid containing aqueous hydrobromic acid in pale yellow prisms or from alcohol mixed with aqueous hydrobromic acid in pale yellow slender prisms (Found. on substance dried at 100° C, C, 52·4, H, 4·2. Calc C, 52 2; H, 4·4 %). Both forms combine normally with secondary bases as is shown below

Dibutylaminomethyl - 2 - *phenyl* - 6 7 - *dimethyl* - 4 - *quinolylcarbinol dihydrobromide* (XIX, $R = $ Ph, $R' = C_4H_{11}$). The preceding carbinol hydrobromide (2·7 g., m.p. 273° C), benzene (22 c.c.) and *n*-dibutylamine (5 c c) were boiled on the water-bath for 14 hr. On addition of excess of dry ether, the precipitated dibutylamine hydrobromide (2·4 g.) was collected and the organic solvents removed from the filtrate. N-Sodium hydroxide (10 c c) was then added and the liberated bases extracted with chloroform. The chloroform was removed and the residual dibutylamine by heating *in vacuo* at 100° C. The residual base was treated with N-hydrobromic acid (10 c.c.) and a little ether to remove non-crystallizable gum The required *hydrobromide* (1·64 g) separated from the mixture in fine short needles. It was recrystallized from boiling N/10-hydrobromic acid, m.p. 210 to 211° C (Found. On air-dried solid, loss at 100° C, 3·2. $C_{27}H_{36}ON_2$, 2HBr, H_2O requires H_2O, 3·1 %. $C_{27}H_{36}ON_2$, 2HBr requires C, 57·1, H, 7·0, N, 4·9. $C_{27}H_{36}ON_2$, 2HBr requires C, 57·2, H, 6·8, N, 4·9 %).

Diamylaminomethyl-2-*phenyl*-6 . 7-*dimethylquinolylcarbinol dihydrobromide* (XIX, $R = $ Ph, $R' = C_5H_{11}$). In a similar way the parent bromomethylquinolylcarbinol hydrobromide (3·35 g., m p. 275°) and *n*-diamylamine (7·5 c c.) gave the required *hydrobromide* (3·64 g) which was recrystallized from boiling N-hydrobromic acid (230 c.c.) and separated in needles, m.p. 207° C (Found: On air-dried solid, dried at

90° C, loss 17·0. $C_{29}H_{40}ON_2$, 2HBr, 7H$_2$O requires H$_2$O, 17·5 %. On dried solid C, 58·5; H, 7·3, N, 4·9. $C_{29}H_{40}ON_2$, 2HBr requires C, 58·5; H, 7·1; N, 4·7 %).

Dihexylaminomethyl-2-phenyl-6 : 7-dimethylquinolylcarbinol dihydrobromide (XIX, R = Ph, R' = C_6H_{13}). The bromomethylquinolylcarbinol hydrobromide (3·0 g., m p. 218° C) *n*-dihexylamine (8·2 c.c) and benzene (30 c c) were heated to boiling for 20 hr. The required base freed from dihexylamine by fractional distillation was treated with N-hydrobromic acid (30 c.c.) in the presence of a little ether and gave the required *hydrobromide* (2·2 g.). It was recrystallized from a mixture of alcohol (10 c.c) and N-hydrobromic acid (5 c.c.) and separated in clusters of needles, m.p. 207 to 208° C (Found: On air-dried solid, loss at 100° C, 4·3, 4·7. $C_{31}H_{44}ON_2$, 2HBr, 2H$_2$O requires H$_2$O, 4·9 %. On dried solid, C, 59·9, H, 7·8; N, 4·7. $C_{31}H_{44}ON_2$, 2HBr requires C, 59·8, H, 7·5, N, 4·5 %).

Morpholinomethyl-2-phenyl-6 · 7-dimethyl-4-quinolylcarbinol hydrobromide. The bromomethylquinolylcarbinol hydrobromide (2·2 g , m.p. 272° C), morpholine (3 c.c.) and benzene (50 c c.) were heated together for 17 hr. N-Sodium hydroxide (10 c.c.) was added and the bases extracted with chloroform. On evaporation of the chloroform and washing with water the required *base* crystallized in buff-coloured prisms, m.p. 165° C (1·6 g.) It was recrystallized several times from benzene and obtained in beautiful colourless prisms, m.p 173° C. Mixed melting-point determinations of the base and hydrobromide with specimens prepared by the alternative method, described below, showed that the two substances were identical.

Piperidinomethyl-2-phenyl-6 7-dimethyl-4-quinolylcarbinol (cf King & Work 1940). Powdered 2-phenyl-6 : 7-dimethyl-4-quinolyl bromomethyl ketone hydrobromide (4·35 g.) was added gradually to a solution of piperidine (3 c.c.) in dry ether (50 c.c.) with continual shaking After 1 hr at room temperature, the piperidine hydrobromide was collected and the ether removed without heating under reduced pressure; during this process the ketonic addition compound crystallized. It was dissolved in methyl alcohol (50 c c) and hydrochloric acid (25 c c., 3N), both of which had been cooled to −5° C, and hydrogenated using Adams's catalyst (0·1 g). Reduction was completed in 5 hr. The catalyst was collected and the mixture evaporated at 50° C under reduced pressure, with frequent addition of a little water to remove excess hydrochloric acid. The reaction mixture was dissolved in water (50 c c.) and extracted repeatedly with ether which removed a gum (1·4 g.) which was shown to be 2-phenyl-6 . 7-dimethyl-4-cinchoninic acid mixed with 4-acetyl-2-phenyl-6 · 7-dimethylquinoline. Further extraction of the aqueous layer with chloroform and evaporation of the chloroform solution yielded a gum (1 3 g.) which was not identified. The aqueous layer was then made alkaline and thoroughly extracted with chloroform The chloroform solution was evaporated, giving *piperidinomethyl-2-phenyl-6 · 7-dimethyl-4-quinolylcarbinol* as an oil (1·05 g.) The *dipicrate* crystallized from methyl ethyl ketone in yellow prisms, m.p. 190° C (decomp.) (Found. C, 53·2; H, 4·2; N, 13·6. $C_{24}H_{28}ON_2$, 2$C_6H_3O_7N_3$ requires C, 52·8, H, 4·2; N, 13·7 %). The *dihydrochloride* crystallized from aqueous alcohol in colourless needles, m.p. 230° C (Found. C, 64·9; H, 6·6; N, 6·5. $C_{24}H_{28}ON_2$, 2HCl requires C, 64·7; H, 6·9; N, 6·5 %) The *dihydrobromide* crystallized from water in colourless needles, m.p. 256 to 258° C (Found: C, 55·2; H, 5·9; N, 5·0. $C_{24}H_{28}ON_2$, 2HBr

requires C, 55·2; H, 5·7; N, 5·4 %). The *base* crystallized from ethyl acetate in colourless needles, m.p. 130 to 131° C (Found: N, 7·7. $C_{24}H_{28}ON_2$ requires N, 7·8 %).

Morpholinomethyl-2-phenyl-6·7-dimethyl-4-quinolylcarbinol was prepared in the same way as the piperidino analogue, except that since the ketonic addition compound was sparingly soluble the morpholine hydrobromide could not be removed in the early stages as was the piperidine hydrobromide, it was therefore separated at the end through its greater solubility in water. The *dihydrobromide* crystallized from aqueous alcohol in needles with a slightly yellow tinge, m p. 236° C (Found. C, 51·0; H, 5·5, N, 5·2 $C_{23}H_{26}O_2N_2$, 2HBr requires C, 52·7, H. 5·4; N, 5·4 %) The *morpholinomethyl-2-phenyl-6:7-dimethyl-4-quinolylcarbinol* crystallized from benzene-*cyclo*hexane mixtures in colourless prisms, m p. 173° C (Found: C, 76·6, H, 7·1; N, 7·6. $C_{19}H_{19}ON$ requires C, 76·2, H, 7·1; N, 7·7 %) The other products of the reaction were the same as in the case of the piperidino analogue, the production of some 4-carboxylic acid again being observed.

Attempts to prepare the dibutyl, diamyl and dihexyl analogues by this method were unsuccessful.

MISCELLANEOUS COMPOUNDS

5·6-Dimethylcarbostyril-4-carboxylic acid was prepared from N-acetyl-4·5-dimethylisatin by the same method as 6:7-dimethylcarbostyril-4-carboxylic acid, described above The yield of *5.6-dimethylcarbostyril-4-carboxylic acid* was practically quantitative, only a very small trace of 4:5-dimethylisatin being recovered. It was purified by dissolving in potassium hydroxide solution and reprecipitating with hydrochloric acid; it formed tiny colourless needles, m.p 356° C (Found: C, 66·4, H, 5·5; N, 6·5 $C_{12}H_{11}O_3N$ requires C, 66·4; H, 5·5; N, 6·5 %). A mixed melting-point determination with the isomeric acid showed a marked depression (330 to 332° C)

2-Chloro-5·6-dimethylcinchoninic acid was prepared by the method used in the preparation of 2-chloro-6:7-dimethylcinchoninic acid, and the yield was the same. The *2-chloro-5·6-dimethylcinchoninic acid* crystallized from glacial acetic acid in colourless cubic crystals, which were, however, anisotropic, m p. 220° C (Found· C, 61·8; H, 4·2, N, 6·1. $C_{12}H_{10}O_2NCl$ requires C, 61·1; H, 4·2; N, 5·9 %). The *sodium salt* crystallized from alcohol in colourless needles, unmelted at 300° C (Found· Na, 8·9 $C_{12}H_9O_2NCl$ requires Na, 8·9 %). A mixed melting-point of the isomeric acids showed a marked depression (202° C).

5.6-Dimethylcinchoninic acid was prepared in the same way as its isomeride, 6·7-dimethylcinchoninic acid. It crystallized from glacial acetic acid in colourless prisms, m p. 284° C (decomp.) (Found: C, 71·3, H, 5·4; N, 7·1. $C_{12}H_{11}O_2N$ requires C, 71·6; H, 5·5; N, 7·0 %)

6:7-Dimethylquinoline. 6.7-Dimethylcinchoninic acid (1 g) was ground with calcium oxide (5 g.) and heated in a distillation flask with a wide side-arm. The 6:7-dimethylquinoline distilled and was purified by sublimation (bath temperature 85 to 100° C/0·3 mm.) and by crystallization from *cyclo*hexane from which it separated in long colourless prisms, m.p. 58° C (Manske *et al.* 1942 give 58° C) (Found: C, 83·8; H, 7·4; N, 9·1. Calc. for $C_{11}H_{11}N$: C, 84·1; H, 7·0; N, 8·9 %). The picrate crystallized from methyl ethyl ketone in short yellow rods, m.p. 269° C (Manske

et al. 1942 give 278° C) (Found: C, 53·0; H, 4·0; N, 14·5. Calc. for $C_{11}H_{11}N, C_6H_3O_7N_3$: C, 52·8; H, 3·6; N, 14·5 %).

5 : 6-*Dimethylquinoline* was prepared from 5 : 6-dimethylcinchoninic acid using the same method as in the case of its isomeride. It was purified by crystallization of the picrate, which separated from methyl ethyl ketone in yellow needles, m.p. 190 to 191° C (Manske *et al.* 1942 give 201° C) (Found: C, 52·8; H, 3·7; N, 14·3. $C_{11}H_{11}N$, $C_6H_3O_7N_3$ requires C, 52·8; H, 3·6, N, 14·5 %). The base prepared from the pure picrate was further purified by sublimation. It crystallized from light petroleum in colourless prisms, m.p 45° C (Manske *et al.* 1942 give 50° C).

Ethyl 6-chlorocinchoninate. This was prepared from 6-chlorocinchoninic acid, for a supply of which we are indebted to Dr T. S. Work, by the action of thionyl chloride and alcohol in quantitative yield. It could be purified by crystallization from ligroin or by distillation in a high vacuum; it was obtained in slender colourless prisms, m.p. 69 to 70° C. Campbell & Kerwin (1946) give m p. 68 to 69° C and record a 75 % yield using alcohol and sulphuric acid for the esterification.

Ethyl 6-chloro-4-quinoloylacetate was prepared using sodamide as described for the 6 : 7-dimethyl compound, the yield (34 %) was not so good as that obtained by Campbell & Kerwin (1946), who used sodium ethoxide and toluene. 6-*Chloro-cinchoninamide* was obtained as a by-product, it crystallized from acetic acid in colourless needles, m.p. 243° C (Found: C, 48·3; H, 4·3, N, 10·4. $C_{10}H_7ON_2Cl$ requires C, 48·0; H, 4·4; N, 10·2 %).

6-Chloro-4-acetylquinoline was prepared by hydrolysis of ethyl 6-chloro-4-quinoloylacetate using acid (as did Campbell & Kerwin 1946) or dilute alkali The former was found to be the better method, alkaline hydrolysis giving rise to a considerable amount of 6-chlorocinchoninic acid.

6-Chloro-4-bromoacetylquinoline hydrobromide was prepared by the method of Campbell & Kerwin (bromination and hydrolysis of ethyl 6-chloro-4-quinoloyl-acetate in chloroform), and also by bromination of 6-chloro-4-acetylquinoline using 40 % hydrobromic acid as solvent (cf. Campbell, Helbing & Kerwin 1946). The yield claimed by Campbell & Kerwin using the first method was not obtained, but the second method was satisfactory The product, m p. 236° C (Campbell & Kerwin give 228 to 230° C decomp.) was obtained in 81 % yield.

6-*Chloro-4-quinolylmethylcarbinol* was obtained in quantitative yield when an attempt was made to prepare α-bromomethyl-6-chloro-4-quinolinemethanol from 6-chloro-4-bromacetylquinoline by a Ponndorf reduction, possibly because heating was continued for 8 hr. The 6-*chloro-4-quinolylmethylcarbinol* crystallized from equal volumes of ethyl acetate and *cyclo*hexane in colourless needles, m.p. 110° C (Found: C, 63·7; H, 4·8; N, 6·7. $C_{11}H_{10}ONCl$ requires C, 63·6, H, 4·8, N, 6·7 %). The *hydro-bromide* crystallized from *iso*propyl alcohol in colourless prisms, m p. 200 to 202° C (Found: C, 45·6; H, 3·9; N, 4·9. $C_{11}H_{10}ONCl, HBr$ requires C, 45·8, H, 3·8, N, 4·9 %).

Quinolinium metho-salts

2-*Phenyl-6 : 7-dimethylquinoline methosulphate* was prepared from the base (1·15 g.) by heating it with freshly distilled dimethyl sulphate (1 c.c.) and benzene (10 c.c.) on the steam bath for 3 hr. The mixture was cooled and diluted with ether,

the required *methosulphate* crystallized in colourless strongly refractive prisms, m.p. 176 to 184° C (1·4 g.) raised to 184 to 186° C by recrystallization from alcohol (Found: C, 63·6; H, 5·9; N, 4·1. $C_{19}H_{21}O_4NS$ requires C, 63·5; H, 5·9; N, 3·9 %). The *methiodide* was prepared by the action of excess solid sodium iodide on an aqueous solution of the methosulphate, it crystallized from methyl alcohol in yellow prisms, m.p. 200° C (gas evolution) (Found. C, 58·0; H, 4·9; N, 3·9. $C_{18}H_{18}NI$ requires C, 57·6; H, 4·8; N, 3·7 %). The *methochloride* was prepared from the methiodide (1 g.) by shaking and warming with a suspension of freshly precipitated silver chloride (2 g.) until the aqueous layer was free from iodide. The silver halide was collected and the aqueous layer evaporated. The residual solid, after crystallization from methyl alcohol-ethyl acetate mixtures, was obtained in colourless prisms, m p. 224 to 226° C, which retained ½H_2O when heated at 110° C (Found· Loss on air-dried material at 110° C 3·0. $C_{18}H_{18}NCl, H_2O$ on losing ½H_2O requires loss 3·0 % Found on material dried at 110° C: C, 73·9; H, 6·5; N, 4·8. $C_{18}H_{18}NCl,$½H_2O requires C, 73·3, H, 6·5, N, 5·1 %).

2-Phenyl-5.6-dimethylquinoline methiodide was prepared from its base through its methosulphate in the same way as its isomeride. It crystallized from methyl alcohol in yellow prisms, m.p 225° C (gas evolution) (Found: C, 58·0, H, 4·9; N, 3·9 $C_{18}H_{18}NI$ requires C, 57·6, H, 4·8, N, 3·7 %). It was also prepared by heating the base with an excess of methyl iodide in a sealed tube at 110° C for 24 hr , but this method offered no advantage. A by-product of this method was the *periodide*, rectangular brown plates with a purple lustre from methyl ethyl ketone, m.p. 190° C (Found. C, 34·3; H, 2·9, N, 2·4. $C_{18}H_{18}NI_3$ requires C, 33·8; H, 2·9; N, 2·2 %).

The *methochloride*, prepared as in the case of the isomeride, crystallized from methyl alcohol-ethyl acetate mixtures in colourless prisms, m.p. 205° C (Found: On air-dried material: C, 71·3; H, 6·9, N, 4·6; H_2O, 6·4. $C_{18}H_{18}NCl, H_2O$ requires C, 71·7; H, 6·6, N, 4·6; H_2O, 6·0 %).

6:7-Dimethylcinchoninamide methiodide was also prepared from its base through its methosulphate, longer heating (12 hr) with dimethyl sulphate was necessary than in the foregoing cases. It crystallized from methyl alcohol in golden orange needles, m p. 256° C (Found. C, 45·6, H, 4·3; N, 8·5. $C_{13}H_{15}ON_2I$ requires C, 45·6; H, 4·4; N, 8·2 %). The *methochloride*, prepared as above, crystallized from methyl alcohol-ethyl acetate mixtures in colourless prisms, m.p 262° C (Found: Loss, on air-dried material, at 110° C, 3·4 $C_{13}H_{15}ON_2, H_2O$ on losing ½H_2O requires, loss 3·3 %. Found on material dried in a vacuum desiccator C, 57·4; H, 6 5, N, 10·3. $C_{13}H_{15}ON_2, H_2O$ requires C, 58·1; H, 6·3; N, 10·4 %).

2-Phenyl-6.7-dimethylcinchoninamide methiodide was prepared from the amide by the same method as its homologue above. It crystallized from methyl alcohol in golden orange needles, m.p. 220° C (Found C, 54·5, H, 4·7; N, 6·6. $C_{19}H_{19}ONI$ requires C, 54·5, H, 4·6, N, 6 7 %). The *methochloride* crystallized from methyl alcohol-ethyl acetate mixtures in colourless prisms, m.p. 263° C (Found: On air-dried material, C, 66·2, H, 6·0; N, 8·1; H_2O, 6·6. $C_{19}H_{19}ON_2Cl, H_2O$ requires C, 66·2; H, 6·1, N, 8·1; H_2O, 5·7 %).

6-Chlorocinchoninamide methiodide was prepared from the amide as above. It crystallized from methyl alcohol in yellow prisms, m.p. 240° C (decomp) (Found:

C, 37·7, H, 2·9, N, 8·1. $C_{11}H_{10}ON_2ClI$ requires C, 37·9; H, 2·9; N, 8·0 %). The *methochloride* also prepared as above, crystallized from methyl alcohol-ethyl acetate mixtures in colourless prisms, m.p. 220° C (gas evolution) (Found: C, 48·3; H, 4·3; N, 10·4, H_2O, 4·0. $C_{11}H_{10}ON_2Cl_2$, H_2O requires C, 48·0; H, 4·4; N, 10·2; H_2O, 6·6 %).

Attempts to prepare the methiodide of 8-bromo-2-phenyl-5 : 6-dimethylquinoline, both by the action of dimethyl sulphate and by heating in a sealed tube with methyl iodide for 24 hr. at 110° C were unsuccessful.

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Fertilization, including chemotactic phenomena in the Fucaceae

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Chemotaxis between gametes particularly of the Fucaceae is discussed and techniques for its detection described.

Cell-free preparations of *Fucus serratus* and *F. vesiculosus* eggs have been obtained which exert a chemotactic attraction on the sperms of *F. serratus*, *F. vesiculosus* and *F. spiralis*, the same chemotactic mechanism among these closely related species being indicated.

The chemotactic principle is easily expelled from aqueous solution by a stream of inert gas, and may be recovered in a cooled receiver; it seems therefore to be a simple compound.

Various arylacetic acids have a marked effect on the *Fucus* sperms studied but this effect is distinct from true chemotaxis and is not related to the plant hormone or herbicide activities of the responsible acids.

INTRODUCTION

The mechanism of sexual reproduction among dioecious organisms has been closely studied biologically and occasionally chemically. A notable contribution from the latter aspect was the recognition of the pigment echinochrome, or at least a near derivative of it, as a factor secreted by the ova of *Arbacia pustulosa* as an attractive agent for the spermatozoids to ensure fertilization (Kuhn & Wallenfels 1939). It should be added that echinochrome is only one of several related pigments (Mayer & Cook 1943) to have been isolated from such organisms, and that the exact function and even the effectiveness of echinochrome appears to require confirmation (Tyler 1939) Perhaps the most impressive if provocative observations in this field concern the production of crocin and *cis*- and *trans*-crocetin dimethyl ester by the gametes of isogamous and anisogamous *Chlamydomonas* species, and the seemingly far-reaching effects of these chemicals on the motility and fertilization of the gametes as well as on their male and female character (Cook 1945).

These observations lent much interest to studies on the pigmentation, particularly of the gametes, of the Fucales (Heilbron 1942). It was found that the male gametes of several of the brown algae including *Ascophyllum nodosum*, *Fucus serratus* and *F vesiculosus* were pigmented almost entirely by β-carotene, whereas that material was lacking in the corresponding female exudates which were coloured mainly by fucoxanthin. On the other hand, the mucilaginous gamete exudates from the hermaphroditic *F. spiralis* contained both β-carotene and fucoxanthin which most probably had been produced by spermatozoids and ova respectively. In seeking a functional explanation of the differing pigmentation it was attractive to assume that as with *Chlamydomonas eugametos* noted above the Fucaceae pigments were related to the specific reproductive functions of the responsible gametes. However,

so little was known unequivocally about chemical control of reproductive processes in the Fucaceae that it was impossible to enlarge this hypothesis immediately, and a return was made therefore to a study of the broader aspects of these phenomena.

METHODS

(a) Freshly gathered whole plants of *Fucus serratus*, *F. vesiculosus*, *F. spiralis*, and *Ascophyllum nodosum* were kept satisfactorily in bulk at 0 to 2° C for periods of up to 1 week, and likewise the severed fruiting tips could be stored with only gradual deterioration except for those of the last-mentioned species, which rapidly fell to a slimy mass. Gamete suspensions were prepared as required merely by steeping the ripe tips in sea water (also preserved by storing at 0° C). By cooling the suspensions of male gametes (from the dioecious members) in ice, the sperms were maintained active for 2 to 3 hr after their liberation from the antheridia.

(b) Drops of the appropriate male and female gamete suspensions were mixed on slides (*Fucus spiralis*, of course, gives a mixed suspension directly) and the fertilization process observed microscopically   Ripe eggs of these Fucaceae exerted a strong attraction on sperms which passed within a distance of 1 to 2 egg diameters (c. 0·1 to 0·2 mm.), thus rapidly collecting about themselves clouds of moving sperms. As a result, these eggs became covered with sperms and were rotated. On fertilization of an egg the action ceased, and closely passing sperms no longer deviated towards it or became attached as a result of chance collisions

(c) After attempts to separate, by means of appropriately narrow strips of filter-paper or by a streak of agar-gel, male and female gamete suspensions placed adjacent to one another upon a slide, the following reliable arrangement was devised. On one side of a microscope slide Apiezon grease was spread thinly, so as to leave uncovered a rectangular portion (c 1·2 × 3·0 cm.) of the glass in the central region. A thin cellophane sheet of dimensions approximating to those of the slide, and which had been creased in half transversely, was pressed on to the greased slide to cover half the area from one end. The crease in the cellophane then lay across the middle of the slide, bisecting the ungreased rectangle. The marginal area of the cellophane was greased, a drop of freshly prepared egg suspension was placed upon the portion of the cellophane affixed to the slide, and the other half of the sheet was folded over about the crease, the greased edges being pressed together. Thus in effect was a thin layer of egg suspension enclosed in a cellophane envelope mounted horizontally upon a slide. Next, upon the uncovered half of the ungreased portion of the glass, a drop of sperm suspension was placed so as to touch the folded end of the cellophane, and the preparation completed with a cover-slip. In further experiments the positions of egg and sperm suspensions were reversed.

It was observed, under the microscope, in the case of *F. serratus* and *F. vesiculosus*, that ripe eggs immediately adjacent to the cellophane partition exerted an attraction on sperms on the other side, since clusters of moving sperms formed (after 5 to 10 min) at positions along the boundary surface nearest to ripe eggs.

(d) One end of a capillary (c. 0·1 × 10 mm.) was inserted into a sperm suspension. The filled capillary was then dropped horizontally into a pool of egg suspension on

a slide, this manner of introduction being adopted in order to prevent capillary flow, either into or out of the tube. Although the eggs were ripe (they responded properly in test (*b*)), no mass movement of sperms took place outwards from the capillary orifices into the egg suspension, and similarly, with positions reversed, there was no general movement of sperms into such a capillary containing ripe eggs. These results can be attributed to the almost complete absence of diffusion at the capillary ends, as was confirmed with coloured solutions.

(*e*) A short capillary tube, one end of which had been drawn out to finer dimensions, was filled with egg extract and mounted horizontally on a slide, with the aid of Apiezon grease, so that the narrow jet was just inserted into a drop of sperm suspension. Slow diffusion of the egg liquor into the sperm suspension took place, shown by the movement of the meniscus in the wider end of the capillary. Sperms were not attracted by the diffusate, rather were they adversely affected by it, becoming immobilized.

(*f*) Female fruiting tips of *F. vesiculosus*, dried in the air for 24 hr, were immersed in sea water, whereupon the liquid became mucilaginous. Equal drops of this solution and fresh sperm suspension were placed adjacent to one another on a slide and then allowed to coalesce By observing the area of junction, it was readily apparent that the mucilaginous solution had a strong immobilizing action on the sperms, those swimming into the solution being brought abruptly to rest

(*g*) Fresh ripe tips cut from female plants of *F. vesiculosus* or *F. serratus* were shaken with sea water (100 c c /100 g. of moist tips) for 10 min. in order to dislodge sexual products from the conceptacles and aid disruption of the oogonial packets and liberation of eggs. The mixture was poured into a glass funnel and the issuing suspension, free from tips, stood for 3 min. The majority of the eggs had then settled and the still-turbid supernatant liquid was decanted, debris thus being removed. After the eggs from 100 g of tips had been washed by decantation with fresh sea water they were transferred to stock bottles with buffered sea water (100 c c) The latter was prepared by mixing sea water (9·5 parts) with a solution (5 parts) made by adding 0·1 M-citric acid (12·2 g /l.) to 0·2 M-disodium hydrogen phosphate (28·4 g./l.) to pH 8·2. The buffering was necessary to prevent the egg suspensions becoming acid and lethal to sperms. The buffered suspensions were kept at room temperature for 24 hr. and then at 0° C. As required, the supernatant liquid was filtered through a fluted paper, and when tested against the sperms by the adjacent drop method (see (*f*)) chemotaxis was exhibited, the sperms rapidly collecting as a clump along the line of junction of the drops.

(*h*) A better method of demonstrating chemotaxis consisted essentially in introducing a minute volume of the solution into the centre of a large drop of sperm suspension spread upon a microscope slide. The apparatus consisted of a bent melting-point tube with one end drawn out to a capillary (*c*. 0·3 mm. diam.), and mounted as shown in figure 1, held in a narrow vertical slit cut in the edge of a small cork disk.

The tube was filled or emptied with the aid of a rubber micro-teat placed on the wider end. A solution to be tested was drawn up the tube to a few mm. above the normal capillary rise. The rubber teat was removed and the tip inserted (as shown)

into the sperm suspension. Slight flow then occurred from the orifice into the surrounding sperm suspension, and chemotactic or other manifestations were readily discernible by observation through the microscope. Chemotaxis, as shown by egg filtrates, was indicated by the formation during 5 to 10 min. of a thick cluster of sperms, individual gametes moving rapidly back and forth within a small area surrounding the capillary orifice (figure 2).

The capillary device itself, filled with plain or buffered sea water, had no more than a transient effect upon the sperms. During the short time sea water diffused from the capillary orifice, most sperms were unable to approach against the current. After a few minutes, however, there was no detectable population difference between this area and areas of the sperm suspension more remote from the capillary. Sperms did not tend to collect in the orifice.
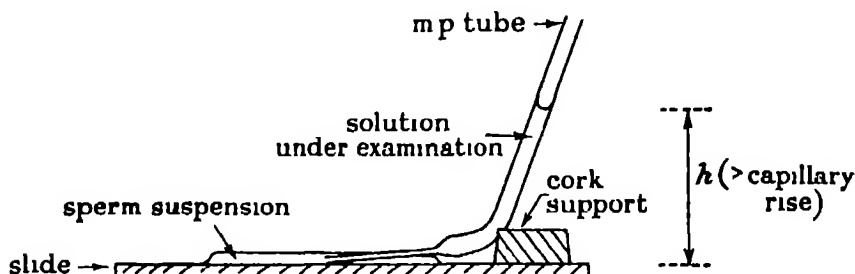
FIGURE 1
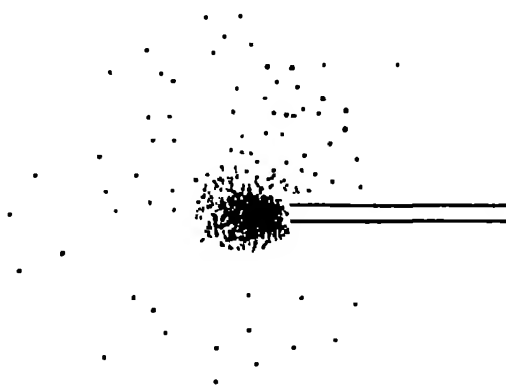
FIGURE 2

(i) A small all-glass bubbler (20 c.c. capacity) containing active egg filtrate (10 c.c.) was aspirated with hydrogen or nitrogen for 3 to 6 min. at the rate of c 2 bubbles/sec. A filtrate which was active up to a limiting dilution of 1:4, when tested by method (h), required a full 6 min. aspiration (at the above rate) for complete loss of activity. Prolonged aspiration was avoided. The bubbler was connected by a short length of glass tubing to a small trap (1·3 × 14 cm.) of the usual pattern, immersed in liquid nitrogen. To aid retention of the active principle it was found necessary to freeze sea water (2 ml.) in a thin film round the sides of the trap.

A similar trap cooled with solid carbon dioxide in ethanol was also satisfactory and possessed an advantage in not condensing as liquid the air which had originally been dissolved in the aspirated sea water. The contents of the trap were tested by method (*h*) after thawing had been allowed to occur.

(*j*) Solutions of certain plant hormones when tested by method (*h*) against sperms of *Ascophyllum nodosum* gave, above certain levels of concentration, a halo of clustered immobilized sperms surrounding the area of diffusion from the capillary orifice. Limiting dilutions for this effect were determined The solutions were prepared by dissolving weighed amounts of the dry sodium salts in measured volumes of sea water, no adjustment of the pH (*c.* 8) being necessary

CHEMOTAXIS IN THE FUCACEAE

It is clear that in view of the prevalent dioecism among these algae and their habitat in moving water, there is likely to be a natural mechanism of attraction rather than mere random coupling of the gametes The absence of such attraction would imply natural hybridization for which the evidence is inadequate, though laboratory experiments have demonstrated the possibility (Fritsch 1945) Chemotaxis has often been considered, though almost all experiments to demonstrate such an influence have been inconclusive. In one instance, however (Kotte 1923), a chemotactic effect was demonstrated between motile sperms of *Fucus serratus* and cell-free mucilage obtained from eggs of the same species. The sperms were more active in presence of the egg preparations and thus seemed to be affected both chemotactically and chemokinetically. Earlier experiments had failed, it seemed, for reasons of technique, and it was concluded that the active factor might well be only slightly diffusible though it appeared to be not excessively stable to heat. Other tactic effects were discerned which, however, probably have little bearing on the present work.

It was obviously desirable in the first place to confirm and extend these observations, working if possible with substantially non-mucilaginous extracts of *Fucus* eggs Several findings, however, soon showed the reality of a chemotactic factor which, unlike that postulated by Kotte (1923), was easily diffusible. In all these experiments, which were carried out entirely in sea water, the suspensions of motile sperms of *F. serratus* (and other species in later experiments) were obtained by immersing the ripe male frond tips in sea water. Not infrequently the sperms proved non-motile and their subsequent behaviour varied considerably and mostly unpredictably, though it was doubtless dependent on the precise state of the fruiting plant. Thus sometimes they gained motility on mild irradiation before a small electric lamp, while on other occasions they remained motionless, partial desiccation of the tips in air followed by moistening with sea water was with some batches sufficient to induce motility, whereas with others sperms were liberated from the conceptacles in intact packets which failed to rupture. It was noticed that in the latter circumstances the sperm pigmentation seemed a light lemon-yellow colour compared with the full orange of the actively motile cells though the absolute difference has not been elucidated.

The not inconveniently mucilaginous suspensions of eggs obtained by simple immersion of the female tips in sea water were shown to attract suitably motile sperms by virtue of a substance which diffused through a cellophane sac; sperms thus clustered on one side of the cellophane interface at those points opposite to eggs on the other side of the interface (see Methods).

The above observations were confirmed using *F. vesiculosus* sperms and eggs, though some complicating factors had to be taken into account. Thus whereas the normal pH of sea water and fresh gamete suspensions was pH 8 to 9, heavy egg suspensions soon acquired a slightly acid reaction (pH 6·8) and a lethal effect on the sperms. The latter effect was lost on adjusting to pH 9, though it was possibly not due entirely to hydrogen ions as the sperms were still able to survive for short periods in sea water which had been acidified to pH 5·6. Again, when other techniques dispensing with a cellophane sac were used and excessive mucilage was not avoided, motility was impeded and observations became erratic. In some instances this hindrance could scarcely have been only mechanical, as some very viscous liquids at pH 7 to 8, obtained by centrifuging heavy egg suspensions or non-fruiting parts of the fronds, caused immediate immotilization of sperms, the effect persisting when the solutions had lost their viscous nature to a large extent by deliberate dilution.

Attempts to obtain cell-free extracts having a chemotactic property by grinding egg suspensions with sand were unsuccessful, but centrifugates of suspensions of *F. vesiculosus* eggs which had stood in sea water for 24 hr. at 0° C were shown by means of a capillary device (see Methods) to attract the sperms of the same species so strongly as to lead to an easily visible yellow cluster round the capillary jet. The effect was often apparent even after four-fold dilution of the centrifugates. Under the influence of such extracts the movement of individual sperms was prolonged though restricted to the small area surrounding the capillary opening. Non-motile immature sperms were not activated by these solutions, which appeared indeed to have a distinctly immotilizing effect on actively moving cells introduced directly into them, rather than subjected to the action of a concentration gradient In the capillary test the sperms only collected in the zone of diffusion and, unless the experiments were very prolonged, did not enter the jet   In short the sperms seemed susceptible with respect to chemotaxis only to a concentration gradient of the active factor (Hoyt 1910) The activity of the solutions was fairly persistent, a sample retaining its effectiveness after at least 10 days at 0° C. On the other hand, the activity was apparently lost by boiling, or by evaporating the solution *in vacuo* even lyophilically.

The extracts of *F. serratus* eggs attracted the sperms not only of the same species but also of *F. vesiculosus* and *F. spiralis*. Moreover, exactly similar experiments were carried out with *F. vesiculosus* eggs which also afforded a cell-free filtrate having a chemotactic attraction for *F. serratus* and *F. spiralis* sperms as well as for those of the species of its origin. The absence of specificity suggests that at least closely related members of the Fucales produce the same chemotactic principle, and the absence of hybridization in nature only emphasizes the importance of other factors in fertilization.

ATTEMPTS TO CONCENTRATE THE CHEMOTACTIC PRINCIPLE

Many of these attempts, for example, to extract the principle of either *F. serratus* or *F. vesiculosus* preparations by means of solvents, were rendered abortive by the apparent destruction of the active principle on evaporation of its solutions; in addition attempts to extract the factor, e.g. by means of chloroform at pH 8, led to loss of activity of the sea water solutions but not to any transference of the activity to fresh water by washing the chloroform 'extracts'.

It seemed that the apparent extreme sensitivity might be due to oxidation, particularly as the activity could be destroyed by sodium periodate solution and apparently by aspirated air. However, all attempts to reactivate solutions, e g. by hydrogen in presence of reducing catalysts, or ascorbic acid, were without useful result. Incidentally the range of reducing agents was rather limited for even 0·002 % quinol, for example, had a pronounced lethal effect on the sperms

The problem assumed a quite different aspect when it was shown that even passage of a moderate stream of hydrogen through an active solution for 2 to 3 min. caused loss of activity. It seemed, therefore, that the active factor was probably a volatile compound having little affinity for water and probably of gaseous nature, though because of ignorance of the quantities involved definite conclusions on the last feature were impossible. By inserting a trap containing a small quantity of frozen sea water cooled in liquid air in the aspirating train, the activity was recovered apparently in undiminished quantity in the condensate. So far therefore from being complex, the active factor must be a quite simple compound, perhaps even inorganic in nature.

A considerable number of simple compounds such as hydrogen sulphide, dimethylsulphide, sulphur dioxide, carbon monoxide and dioxide, ammonia, methylamine, formaldehyde, oxygen, ozone, simple olefines and nitrogen oxides were tested empirically over a wide range of dilutions. Only in one isolated case, however, with aqueous solutions of nitrous oxide at a limiting dilution of about 1 20,000,000, was any positive effect observed. On this occasion only sperms of *F. spiralis* which were in an obviously enfeebled condition were available and as the experiment was not reproducible it is of questionable significance.

THE EFFECT OF ARYLACETIC ACIDS ON *FUCUS* SPERMS

The possibility was considered at an early stage in the above work of the chemotactic action being related to an unidentified growth hormone present in *F. vesiculosus* eggs (du Buy & Olsen 1937). It is now clear that the physical nature of the chemotactic factor precludes this possibility, but in examining some compounds known to have activity as plant hormones it was found that crystals, of dimensions approximating to those of *Fucus* eggs, of β-indolylacetic or α-naphthylacetic acid had a surprising effect on the sperms of *Fucus* species. When placed in sperm suspensions the crystals quickly collected dense clusters of sperms by virtue of an immotilizing action. The action is thus quite different from the

chemotaxis examined above, which is a result of control on the direction, rather than on the degree, of movement of the sperms. On removing the crystals, motility was regained and the sperm cloud largely dispersed. The effect was not due merely to acidity for benzoic, phenylacetic, hippuric and phenaceturic acids were ineffective, the first two being highly lethal to the sperms. Moreover, neutral solutions of the sodium salts of α-naphthyl- and β-indolyl-acetic acids in sea water had the same cluster-forming effect as the free acids. By means of the capillary device (see Methods) the 'chemotaxis' which resulted in a halo of sperms in the neighbourhood of the capillary tip could be assayed semi-quantitatively, limiting dilutions using *F. vesiculosus* sperms were 0·3 and 0·02 % for the two acids respectively. It is a remarkable circumstance that 0·01 to 0·1 % solutions of potassium β-indolyl-acetate had been apparently incidentally observed as long ago as 1884 to have an inhibiting action on sperms of *F. serratus* (Pfeffer 1881–5).

The effect of a number of other arylacetic acids including some potent plant hormones and weed-killers were tested by the capillary technique using sperms of *Ascophyllum nodosum* which is closely related to Fucaceae already discussed. Several of these hormones had also a sperm-collecting action, the limiting dilutions being shown below:
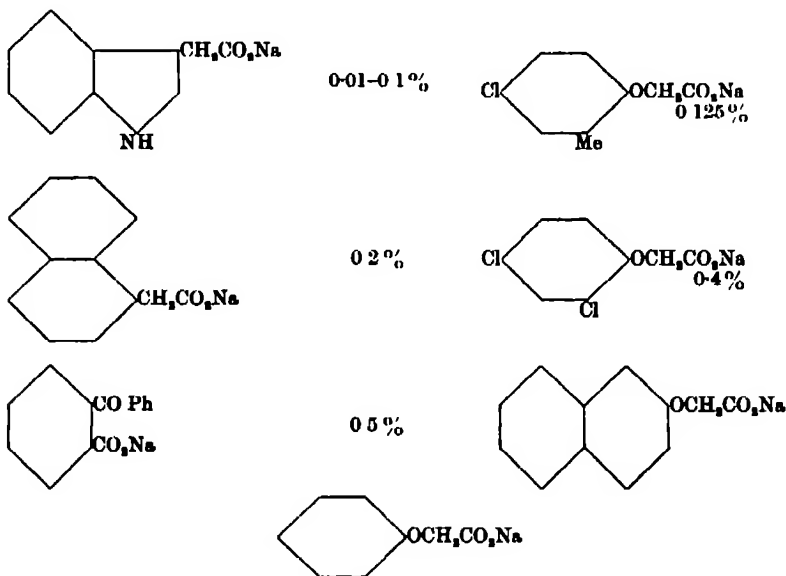
No connexion between plant hormone or herbicide activity on the one hand and sperm inactivating effect on the other could be traced.

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Adjustments in bacterial reaction systems
I. The reducing power of *Bact. lactis aerogenes* under various conditions

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The reducing power of cells of *Bact. lactis aerogenes* has been measured by dye reduction tests with resting and with growing cultures in media containing as nitrogen sources ammonium salts or nitrate, and under aerobic and anaerobic conditions

Changes in growth rate have been correlated with changes in the reducing power accompanying transitions from one medium to the other or from anaerobic to aerobic conditions and vice versa.

Two types of adjustment play a part in the observed phenomena· (*a*) rapid changes in concentrations of active intermediates, (*b*) slow adaptive modifications of enzyme systems in response to needs of the cell.

The former are exemplified *inter alia* by the rapid fall in reducing power and growth rate when an anaerobic nitrate culture is aerated The latter include the development of extra reducing power when the cell has to use nitrate as a source of nitrogen and of still more when the nitrate has to be reduced in presence of oxygen

1. Introduction

Bacterial cells can live and function under an extraordinarily wide range of conditions, deriving their material and their energy from very varied sources When the conditions are changed, however, transitional phenomena of great interest appear. These include, on the one hand, lags and actual arrests of growth occurring while certain kinds of adjustment are in progress, and, on the other, the adaptive processes whereby growth in new conditions gradually leads to optimal functioning.

Previous work in this laboratory has touched upon these themes in different ways. Adaptive mechanisms have been studied in the light of the hypothesis that adjustment to utilize new sources of material or to resist drugs involves a change in the proportions of various parts of the cell substance (Davies, Hinshelwood & Pryce 1944; Lodge & Hinshelwood 1944; Postgate & Hinshelwood 1946). In particular, *Bact. lactis aerogenes* and *Bact. coli*, normally grown with ammonium salts as the nitrogen source, were shown to adapt themselves to the optimum utilization of nitrate and nitrite by serial subculture in media containing these salts. The reduction mechanisms appeared to be closely linked with the normal dehydrogenase systems of the cells (Lewis & Hinshelwood 1948). In the subsequent development of this work the following observations were made. When a culture is growing with reduction of nitrate or nitrite and an ammonium salt is added to the medium, the reduction ceases abruptly and, in general, only begins to show a slow and autocatalytic recovery when the ammonium salt is nearly all consumed. When a culture is growing in a nitrate medium through which a stream of nitrogen is passed to maintain anaerobic conditions, and the nitrogen is replaced by air, growth ceases abruptly and only begins again after a considerable delay.

These facts received a general interpretation in terms of the hypothesis that growth in the normal medium, with glucose as the source of carbon, involves an oxidation-reduction system X, XH_2, which is common to more than one series of reactions. The ratio $[XH_2]/[X]$ expresses the reducing power of the cell. Sudden aeration lowers this ratio and so impedes nitrate and nitrite reduction. Optimum growth with utilization of ammonium salts demands a low value of the ratio (as shown by the need for effective aeration). Thus optimum growth in ammonium salts is not compatible with reduction of nitrate or nitrite

These investigations have now been followed by direct experiments on the reducing power shown by the cells under various conditions of growth. The results have bearing upon the general question of the co-ordination of cell mechanisms. They receive a satisfactory interpretation in terms of the view that there occur two kinds of adjustment, namely, rapid changes in the concentrations of active intermediates, and slower enzymic modifications which can only be completed by actual growth.

2. Reduction of dyes by cell suspensions. Methylene blue

The first part of the experimental investigation was necessarily devoted to the study of the reduction of dyes by cell suspensions with the object of finding suitable tests for the measurement of the reducing power at various stages of growth in different kinds of media. The point of departure was the well-known Thunberg methylene-blue reduction test (cf Hewitt 1936)

In some of the experiments the solutions used were saturated with air · in others the air was replaced by bubbling through the solutions a stream of nitrogen very thoroughly freed from oxygen by passage over reduced iron at 700° C in an apparatus due to Dr B. Lambert (to whom we are indebted for its loan). In some experiments the reduction of the dyes was gauged by comparison with visual standards, and

in others the solution under test was transferred to the glass cell of a Spekker absorptiometer and the reduction estimated colorimetrically. When the conditions were to be kept anaerobic the cell was covered with a greased glass plate, care being taken that no bubbles were trapped inside. Where continuous readings were required of the fading of the dye, the Spekker instrument was enclosed in a glass case kept within a degree or two of 40° C, the culture solutions being maintained at 40·0° C in a thermostat.

The cells used were from a strain of *Bact lactis aerogenes* maintained in the laboratory. Media consisted of phosphate buffer, magnesium sulphate, glucose and the nitrogen source as described by Lewis & Hinshelwood (1948) The preparation of suspensions was as described by previous authors (Quastel & Whetham 1924, 1925; Davies & Hinshelwood 1947).
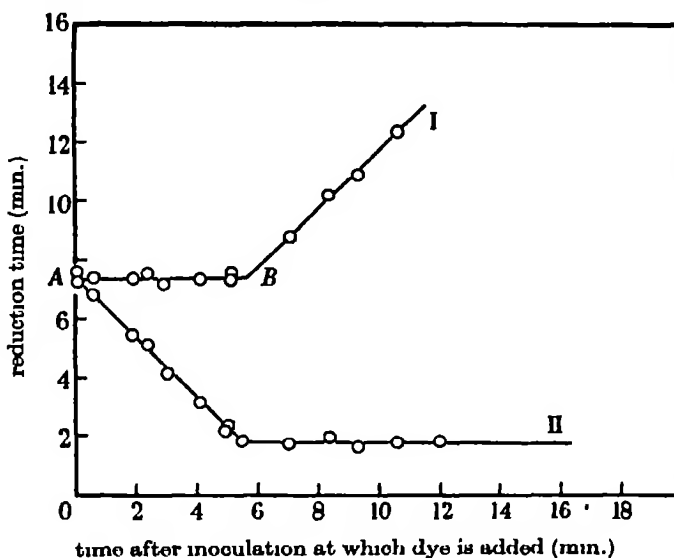
FIGURE 1. Reduction of methylene blue added after inoculation. I. Reduction time measured from inoculation II. Reduction time measured from addition of dye.

It was first established that the time required for 50 % reduction of methylene blue is made up of two parts, the first being independent of the dye concentration and determined simply by the removal of oxygen from the system, the second representing the reduction of the dye itself This may be shown in various ways.

A series of media each consisting of 5 ml of the normal growth medium minus the nitrogen source were simultaneously inoculated with 1 ml. of a suspension of washed cells. At any required time 0·2 ml. of a 1 g /l. solution of methylene blue was added so as to give a concentration of 33·3 mg /l. Air in the space above the solution was replaced by nitrogen. The time required for the dye to be half reduced was measured. Typical results are shown in figure 1.

The time from inoculation to the chosen reduction end-point is independent of the moment of addition of the dye, provided that it is added before the point *B* From *A* to *B* some process is occurring at a rate independent of the presence of the

methylene blue. If the dye is added after B, its own time of reduction is constant, as shown in the lower curve of figure 1.

The length of AB is a function of the initial oxygen content of the solution, as shown in figure 2, and thus AB is almost certainly concerned with oxygen removal. The time required for the second part of the reduction process is directly proportional to the concentration of the dye itself, and thus this phase represents the actual dye reduction. This is shown by the linear plots in figure 2.
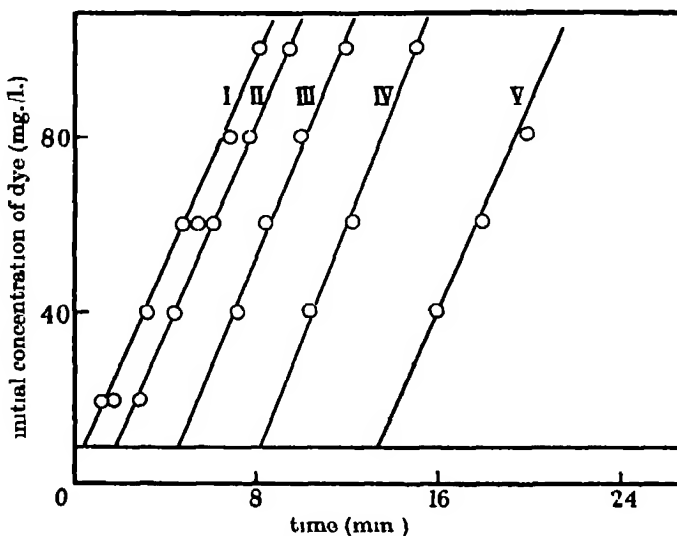
FIGURE 2. Effect of oxygen on duration of first phase of reduction, and of initial concentration of dye upon second phase I. Dye added after removal of oxygen by cells II. Complete exclusion of oxygen as far as experimentally possible III. Media boiled; cell suspension oxygen-free IV. Media boiled, suspension saturated with air at 40° C V. Media and suspension saturated with air at 40° C.

Careful experiments were next made with a growing culture saturated with air at 40·0° C. Methylene blue was added to give a concentration of 60 mg /l., the aeration was stopped and a sample was transferred to the cell of the absorptiometer, all further access of air being prevented by the ground-glass cover. The fading of the dye was observed continuously, and from a calibration curve the precise concentration of that remaining at each time was found No reduction of the methylene blue occurred for 5·52 min. At the end of this time the concentration began to fall at a rate of 23·0 mg /l./min. This rate remained nearly constant until the reaction was about half complete and then slowly decreased.

The concentration of oxygen in a solution in equilibrium with air at 40·0° C is 0·0062 g /l. or 0·775 mequiv./l. This corresponds to 124 mg /l. methylene blue. The removal of the oxygen required 5·52 min., which corresponds to a rate of dye removal of 22·5 mg./l./min., which is close to the observed rate of methylene-blue reduction of 23·0 mg /l./min

It appears, therefore, that oxygen and methylene blue are reduced at equivalent rates.

3. COMPARISON OF THE BEHAVIOUR OF VARIOUS DYES

The dyes investigated are given in the following list; not all of them proved suitable for quantitative work:

dye	E'_0 at pH = 7 0
Bindschödler's green	0 224
1-naphthol-2-sodium sulphonate indophenol*	0·123
tolylene blue	0·115
Lauth's violet	0 063
brilliant cresyl blue	0 047
methylene blue	0 011

* This will be referred to for brevity as 'naphthol pink'.

The oxidizing power of the dyes decreases from top to bottom of the list.

The results illustrated in figure 3 were obtained with washed cells in the glucose-phosphate-magnesium sulphate medium without nitrogen source, the solution containing a small amount of air derived from the cell suspension and the air above the test solution having been replaced by nitrogen. The reduction time was determined for various cell counts and its reciprocal plotted as in the figure From this may be read off the reduction time for a cell count of 150 units (the unit is 10^6/ml). The numbers thus found are given below.

redox potential	reduction time (min)
0 224	1·3
0 123	1 9
0·115	2 1
0 063	3 1
0 047	3·4
0 011	3 6

In other words, increase in the oxidizing potential of the dye is accompanied by a general increase in the speed of reduction, though the differences between the three lowest numbers are small. This will be referred to as behaviour of type I The relations, however, are by no means as simple as this might suggest, and depend upon the conditions of the experiment.

If the above tests are repeated with an aerated medium, the three dyes lowest in the above oxidation scale are not decolorized at all The three more easily reduced dyes are in fact reduced, though in times considerably longer than those found in the anaerobic tests, but after some time they are reoxidized once more. This shows that as the aeration of the medium is continued the actual balance of cell processes in the resting culture undergoes a change.

The complication revealed by this last observation appears in a more general form on further investigation. According to conditions, two types of behaviour are observed: type I, already exemplified, where the reduction time is less for dyes whose potentials suggest easier reduction, and type II, where a quite different relation holds. Type I tends to appear when cells are taken from an actively growing culture and to give place to type II when they are tested some time after inoculation into a medium lacking a nitrogen source for growth. This indicates
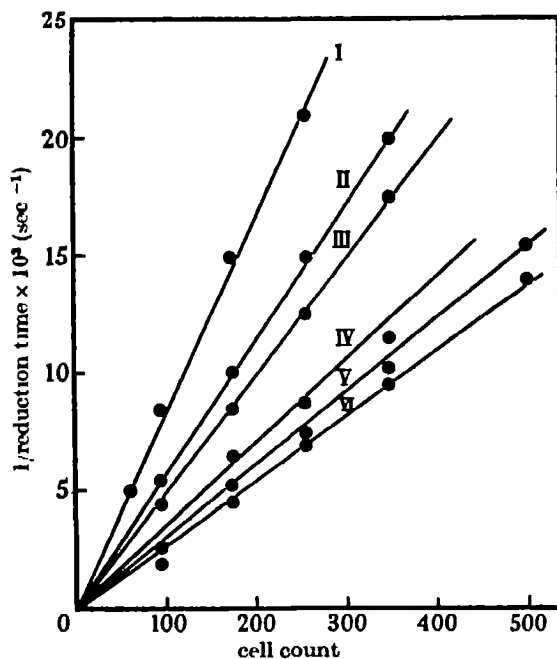
FIGURE 3. Reduction time and cell count. I. Bindschedler's green. II. 'Naphthol pink' III. Tolylene blue. IV. Lauth's violet. V Brilliant cresyl blue VI Methylene blue
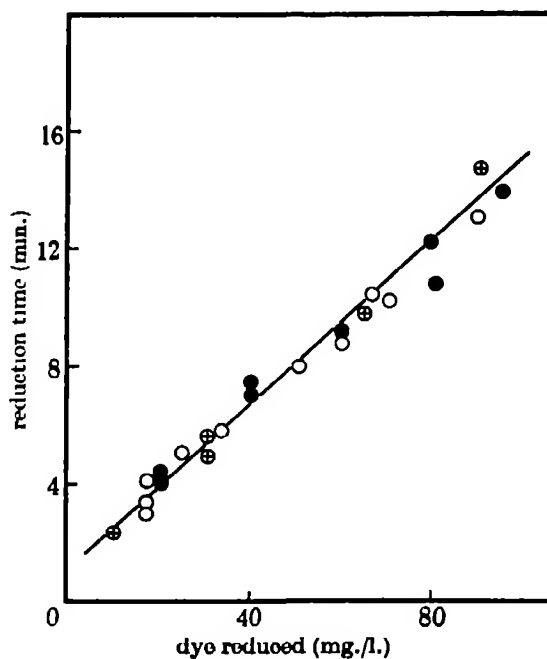
FIGURE 4. Reduction of dyes by washed cells (type I behaviour) in absence of air. Open circles, methylene blue. Crossed circles, brilliant cresyl blue. Black circles, 'naphthol pink'.

once more that the balance of cell processes can shift in such a way that the dyes whose potentials are far above that of methylene blue are no longer accessible to attack.

Under the appropriate type I conditions 'naphthol pink', brilliant cresyl blue and methylene blue *in the absence of air* are all reduced by a washed suspension, also saturated with nitrogen, at the same rate. This is shown by the results in figure 4.

If the solutions are initially saturated with air, Lauth's violet, brilliant cresyl blue and methylene blue give nearly the same reduction times, but with 'naphthol pink', and with Bindschedler's green, the reduction times are shorter, some reduction occurring while air is still present. The contrast in behaviour between 'naphthol pink' and methylene blue is shown in figure 5.
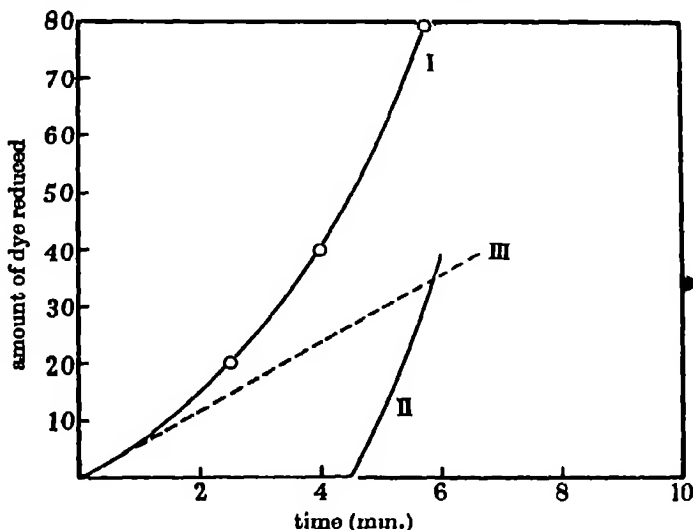
FIGURE 5. Reduction of methylene blue and 'naphthol pink' by cells showing type I behaviour. I. 'Naphthol pink'; air present initially. II. Methylene blue; conditions as for I. III. 'Naphthol pink'; aerobic throughout.

Under type II conditions in anaerobic tests with 60 mg./l. of dye the rates of reduction are, initially and over a wide range, 14·5 % per min. for methylene blue, 13·9 % per min. for brilliant cresyl blue and 3·6 % per min. for 'naphthol pink'.

Figure 6 shows the reduction of methylene blue and of 'naphthol pink' in media initially saturated with air, with cells in the type II condition. The reduction of the latter dye is seen to set in sooner but to proceed more slowly.

As to the interpretation of the two types of behaviour, all that can be said at the moment is the following. In optimum (type I) conditions, all the dyes appear themselves to be reduced anaerobically at the same rate, and those of higher oxidizing potential can even compete with oxygen, so that aerobically the final reduction may be attained more quickly than with those of lower potential. As the cells pass into the condition favouring type II behaviour, mechanisms which are involved in the reduction of the dyes of higher potential (remote from methylene blue in the series) appear to pass out of action. This suggests the existence of

a cascade of oxidation-reduction processes which when fully operative can transfer hydrogen to any of the dyes, but parts of which become inoperative in resting cells, so that these can only transfer hydrogen to dyes appropriately placed in the series (and, in point of fact, not too far removed from methylene blue).
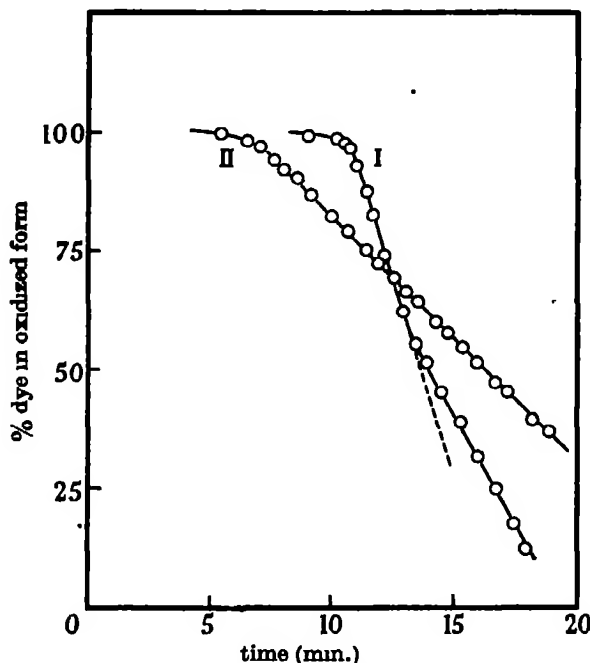
FIGURE 6. Reduction by washed cells in a solution initially saturated with air (type II behaviour). I. Methylene blue. II. 'Naphthol pink'.

For our present purpose there is no need to pursue this matter, but it will be further investigated in connexion with experiments on the utilization of different carbon sources in aerobic and anaerobic conditions. The above experiments are enough to define the conditions of tests applicable to the study of nitrate reduction and similar phenomena. The behaviour with different dyes obviously depends in a rather complex way on the various oxidizing and reducing functions of the cell in its different states. For the study of nitrate reduction it was therefore decided not to rely upon the order of the dyes in the redox series but to make comparative tests, aerobically and anaerobically, in different media and under various conditions with one or two standard dyes without relying upon the relation between the dyes themselves.

4. ROUTINE TEST FOR EXAMINATION OF CULTURES

For the study of the reducing power of various strains of bacteria in nitrate and in ammonium sulphate media under different conditions some standard tests were necessary. In the light of the foregoing the following were adopted:

(a) *Aerobic test.* A 5 ml. sample of the culture was withdrawn and kept aerated in a 6 × 1¼ in. test-tube at 40·0° C. 0·2 ml. of a 1 g./l. solution of methylene blue was

then added. The air supply was cut off and the tube twice evacuated and filled with nitrogen. The time between the cutting off of the aeration and the moment when the methylene-blue concentration fell to 5 mg./l. (estimated visually) was noted. During this time approximately 1 mequiv. of hydrogen has to be released for reduction of oxygen and dye.

(*b*) *Anaerobic test.* A 5 ml. sample of the culture was transferred to a test-tube through which a slow stream of pure nitrogen was passing. 0·4 ml. of the 1 g./l. solution of methylene blue was added and the test-tube immediately evacuated twice and refilled with pure nitrogen. The reduction time was then measured as before. In this test approximately 0·5 mequiv. of hydrogen has to be released for reduction of the dye.

The activity of the cells is expressed as 1/(reduction time) divided by cell count and, for convenience, multiplied by 10^4. Activities in the anaerobic test are normally halved for comparison with the aerobic test, since only half as much reduction has to be accomplished.

Both tests could be varied by substitution of 'naphthol pink' for methylene blue.

5. REDUCING POWER OF CULTURES DURING LOGARITHMIC GROWTH

The reducing power of actively growing cultures in the ammonium sulphate medium and in the nitrate medium was examined both by the aerobic and by the anaerobic test. The results are given in table 1.

TABLE 1. RELATIVE REDUCING POWERS (RECIPROCAL REDUCTION TIMES)

dye reduced	methylene blue	brilliant cresyl blue	Lauth's violet	'naphthol pink'
E_0'	0·011	0·047	0·063	0·123
AEROBIC TEST				
medium				
series I				
aerobic ammonia	15 0	—	—	22·5
aerobic nitrate	22·5	—	—	31·5
series II				
aerobic ammonia	15·3	15·2	14·8	18·1
anaerobic ammonia	14·8	14·4	14·9	18·5
aerobic nitrate	22·3	22 0	—	24·8
anaerobic nitrate	0	0	—	19·8
ANAEROBIC TEST				
anaerobic ammonia	14 6	14·9	—	14·8
anaerobic nitrate	0	0	5·0	10·3

Figure 7 shows that throughout the logarithmic phase in a given medium a constant level of reducing power is maintained.

From table 1 the following conclusions may be drawn about this reducing power: (*a*) In the ammonium sulphate medium it is approximately the same whether the culture is aerated or not. (*b*) In aerated nitrate cultures it is greater than in the corresponding ammonium sulphate cultures. (*c*) In the nitrate medium it is greater for aerobic than for anaerobic cultures.

Translating these results into terms relevant to the general discussion we may say:

I. The reducing power needed and developed by the cell in ammonium salt-glucose media is nearly the same for aerobic and for anaerobic growth.

II. In nitrate media the cells develop the extra reducing power which they need in order to obtain their nitrogen from the nitrate.

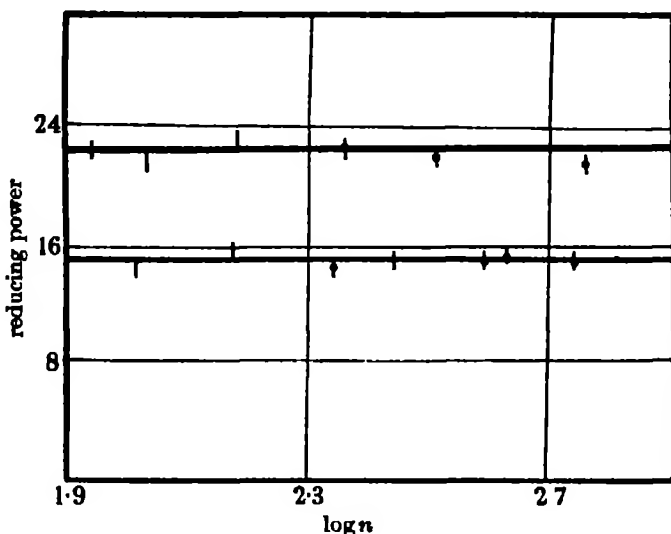III. That to reduce nitrate in presence of oxygen the cells need and actually develop even more reducing power than they need and develop in its absence.

FIGURE 7. Constancy of reducing power during logarithmic growth. Upper line, 'naphthol pink'. Lower line, methylene blue. (The 2 ml. samples (♦) were diluted to 5 ml. (|) with fresh medium for the test.)

6. REDUCING POWER DURING INITIAL STAGES OF GROWTH

When washed cells taken from an actively growing aerobic culture in the glucose-ammonium salt medium are placed in a medium lacking a nitrogen source, they show at first the behaviour characteristic of their original condition, giving values of 15·0 for methylene blue and higher values for 'naphthol pink'. After a time, however, even as little as 10 min , the reduction time for the latter rises and the cells show the behaviour referred to as that of type II.

If ammonium sulphate is added about 30 min. after inoculation, growth starts at once at the optimum rate. As growth proceeds, the rate of reduction of the 'naphthol pink' slowly increases, that of the methylene blue showing no detectable change.

When sodium nitrate is added as the source of nitrogen, the growth does not begin at once. Under anaerobic conditions there is a delay of about 15 min. and within about 30 min. logarithmic growth is established. There is, during this initial period, a rapid decrease in the ability of the cells to reduce the methylene blue; no reduction at all occurs when the logarithmic phase is established.

When the nitrate is added to an aerated culture there is no growth at all for over 2 hr., during which time the reduction rates of methylene blue and of 'naphthol pink' slowly decrease. Slow growth then sets in and the reducing power towards both dyes rises. About 5 hr. after the addition of the nitrate, logarithmic growth, with mean generation time 52 min., is established, and the reducing power towards the dyes has risen to the values characteristic of the steady state (table 1). The growth rate and the reducing power are closely parallel as can be seen in figure 8.
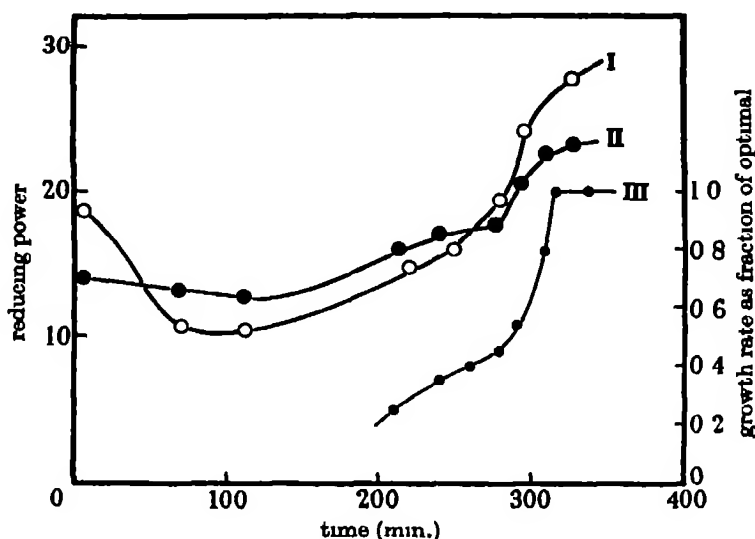
FIGURE 8. Development of reducing power after addition of nitrate to aerated suspension Correlation of reducing power and growth rate I. 'Naphthol pink'. II Methylene blue. III. Growth rate

From these results we derive the following conclusions which for future reference are numbered consecutively with those of the previous section.

IV. Steady conditions for the anaerobic reduction of nitrate are established very much more rapidly than those for the aerobic reduction, the greater reducing power required for the latter being developed only in the course of growth.

V. Steady conditions for optimum growth in the ammonium salt medium are established rather rapidly

VI. Slow and reversible processes occur in resting cells which cut out the reduction of 'naphthol pink' while leaving that of methylene blue unchanged. This has already been discussed in §3.

7. EFFECT OF CHANGING AERATION CONDITIONS DURING GROWTH

During logarithmic growth, conditions were changed from aerobic to anaerobic or vice versa, the cell counts and the reduction times for methylene blue and for 'naphthol pink' being determined at frequent intervals. The results are given in table 2.

TABLE 2

medium	transition	effect observed
ammonium salt	anaerobic to aerobic	no arrest in growth. Mean generation time changes from 42 to 32 min. within 10 min. No significant change in reduction times
	aerobic to anaerobic	arrest of 20 to 30 min. after which logarithmic growth with mean generation time of 42 min. is established. Reduction time steady
nitrate	anaerobic to aerobic	long arrest and slow recovery, dye reduction and growth rate increasing in parallel to their final values
	aerobic to anaerobic	no arrest. Mean generation time changes from 52 to 42 min. in the course of about 20 to 30 min. The ability of the culture to reduce methylene blue rapidly decreases to zero

The conclusions from these experiments are:

VII. Aerobic growth is more rapid in ammonium salt medium, anaerobic growth in nitrate.

VIII. During anaerobic growth in ammonia the enzyme systems necessary for aerobic growth seem to be fully mobilized, but during aerobic growth some of those needed for anaerobic growth are not, and need 20 to 30 min. for mobilization The aerobic-anaerobic transitions with ammonium salts are, however, less drastic than with nitrate.

IX. The deleterious effect of oxygen on growth in nitrate is exerted at once, the recovery requires very considerable adjustments which are only completed during growth.

X. Growth in nitrate and reducing power run parallel.

8. EFFECT OF AMMONIA ADDITIONS TO AN AERATED NITRATE CULTURE

Addition of ammonium sulphate to a growing aerobic culture in nitrate has already been shown to cause increased growth rate (with utilization of the ammonia) and a sharp fall in nitrate reduction (Lewis & Hinshelwood 1948). The accompanying changes in reducing power have now been studied. Figure 9 shows that on addition of the ammonium sulphate there is a fall in the manner which might be expected from the results of the preceding sections.

When the ammonia has been used up, growth in nitrate recovers and the reduction rates increase correspondingly. (Recovery under these conditions requires considerably longer than the time taken by washed cells in nitrate to reach optimum growth, the growth in ammonia leading apparently to some specific inhibition of the recovery process.)

The result of this section is to show that:

XI. When growth in nitrate is arrested by addition of ammonia the reducing power drops.

9. TRAINING OF CELLS TO AEROBIC GROWTH IN NITRATE

It has already been shown that serial subculture in media containing nitrate is accompanied by adaptation of the cells to utilize the medium more effectively. The question now arises as to the corresponding changes in the dye reduction test.
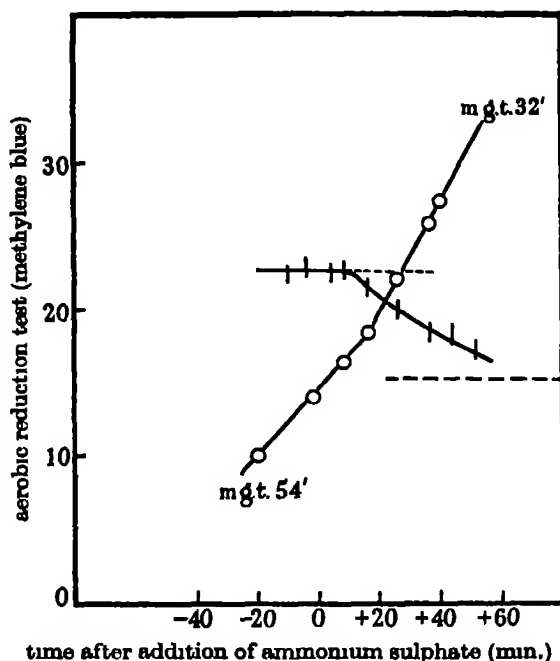

FIGURE 9. Addition of ammonium sulphate to growing nitrate culture. Open circles, growth curve. Vertical strokes, reducing power. The dotted lines show the values characteristic of nitrate and ammonium sulphate cultures respectively.

Normal cells were grown for the first time in the nitrate medium, and were subjected to the aerobic methylene blue test. After growth the cells were washed and reinoculated into nitrate. This was repeated through a series of subcultures. The results are given in table 3.

TABLE 3

strain	reducing power		lag (min.)	mean generation time	
	washed cells	growing cells		aerobic (min.)	anaerobic (min.)
untrained	14·8	22·7	270	54	44
3 subcultures	17·2	22·3	160	52	42
32 subcultures	22·3	23·2	50	49	39

As the cells adjust themselves to optimum growth in nitrate the concentration of certain reducing substances tends towards higher values. Dilution of the culture during growth seems able to cause loss of diffusible material and to impair the reducing power as shown in table 4.

TABLE 4

stage of growth expressed as number of cell divisions which have occurred in nitrate	aerobic methylene-blue test	
	diluted sample	undiluted sample
0	15·0	15 0
1	15 5	22·0
2·5	18 6	22·6
4	20·3	22·3

The culture is, however, better able to withstand the effect of dilution the longer growth in nitrate has continued, there being then presumably a reserve of enzyme activity.

From the results of this section we conclude·

XII. That although a moderate amount of cell multiplication in the nitrate medium is enough to evoke the increased reducing activity needed for aerobic growth, the stabilization of this increased activity demands serial subculture for some time. This is parallel with what is found during training to resist drugs or during training to utilize new sources of carbon (Hinshelwood 1946). There are various interpretations possible. Whether in this instance the final stable enzyme system is the same as that responsible for the initial reversible increase in activity cannot be stated decisively.

10. DISCUSSION

The proximate conclusions of each of the previous sections have been given in the appropriate places. It remains to consider their interpretation. (The references are to the numbered conclusions of previous sections.)

Certain of the facts receive a simple explanation in terms of the variations in concentration of an intermediate common to more than one set of enzyme processes. We write the scheme (in which there may be more than one compound of the type XH_2, and where the processes schematized by (1), (2), and (3) may be complex):
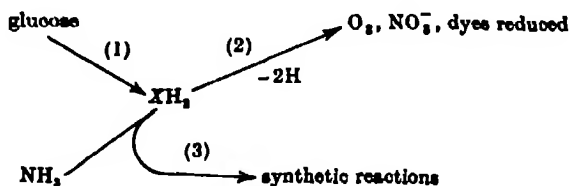
In logarithmic growth in a given medium a steady concentration and a steady rate of production of XH_2 is established, which is reflected in the constancy of the dye test. In the nitrate medium, XH_2 attains a higher concentration than in the ammonium salt medium. If oxygen is passed into an anaerobic nitrate culture $[XH_2]$ is rapidly lowered and the growth rate and reducing power drop (IX and X).

In nitrate media the cells can obtain no nitrogen for growth except by reducing the nitrate. Oxygen, by keeping $[XH_2]$ lower than it might otherwise be, lowers growth rate. In the ammonium salt media there is no such deleterious effect of oxygen, and the more complete oxidative processes possible in these media make aerated growth more effective than anaerobic. The sequence of growth rates is thus: aerobic growth in ammonia, anaerobic growth in nitrate or ammonia, aerobic growth in nitrate (VII).

The addition of ammonia to a nitrate culture causes a diversion of XH_2 with immediate drop in nitrate reduction (XI). As soon as aeration is stopped in a nitrate medium, the removal of the competition by oxygen for the intermediate XH_2 allows the latter rapidly to reach a higher concentration and the growth rate rises (IV).

In all these examples we have to deal with a very rapid response, the adjustment being simply that of the concentration level of an active intermediate in the oxidation-reduction system.

The other conclusions listed in the previous sections relate to more profound adjustments in enzyme systems, which arise in response to definite needs of the cell. These are slow, and, in principle, may include both expansions of particular enzymatic regions of the cell by actual growth, and slow accumulation of necessary co-enzymes in processes analogous to those occurring during lag phases.

For the utilization of nitrate, extra reducing power must be developed, and in fact does appear, but only slowly. For aerobic utilization the reducing power needed is higher (because the competition of oxygen has to be overcome) and develops more slowly still (II, III, IV, XII)

One observation calls for special comment. Although it is consistent with the whole interpretation given that methylene blue should be less easily reduced in the anaerobic nitrate cultures, it is surprising that in these conditions no reduction should occur at all. The explanation is probably of a specific nature In these cultures the nitrate has to be reduced not merely to provide *nitrogen* but to replace *oxygen* for quite other purposes. This may well involve adjustments in various oxidizing systems and the resulting change in the balance of reactions may lead to the formation of intermediates which can actually reoxidize leuco-methylene blue.

As to the relation of aerobic and anaerobic growth in ammonium salt media, it has appeared that little adjustment is needed in the transition from the latter to the former. Certain extra powers have, however, to be mobilized for the reverse transition, and require a little time. They do not, however, seem to affect the reducing power which is about the same for the cells adjusted to either condition (I, V, VIII).

The various adjustments discussed fall into two classes: those due to concentration changes and those depending upon a modification in overall enzyme activity which arises in response to a need. The former require no further explanation. The latter, which are the more strictly adaptive changes, have been shown to play an important part in the particular phenomena described. They are, however, of quite general importance and their mechanism is discussed in correspondingly general terms in the following paper.

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Adjustments in bacterial reaction systems

II. Adaptive mechanisms

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General propositions about the way in which enzyme systems can adapt themselves to respond to new needs of bacterial cells are considered

In certain conditions the establishment of an optimum growth rate is automatic. In the simplest treatment the 'enzyme' material is regarded as autosynthetic, but it is shown that similar equations may hold where the synthesis of one enzyme is linked with the working of another enzyme rather than with its own

1. Introduction

In the study of cell mechanisms three kinds of adjustment play their parts. The first is the rapid alteration in the concentration of an active intermediate involved in the reaction sequence. This can account for sudden arrests of growth and similar effects which follow upon a change in the conditions Examples have been given in the preceding paper. The second kind is the slow accumulation of necessary intermediates, co-enzymes and so on, and is of special importance in the study of the lag phase. The third is the type of adjustment whereby, during continued growth, the enzyme systems of the cell change in such a way as to respond to new needs and to establish an optimum rate of multiplication. This type of response was exemplified in the preceding paper by the development of reducing power needed for growth in nitrate. Its mechanism is to be considered in a more general way in the present paper.

During growth all the cell enzymes are reproduced. The final result of the co-ordinated cell reactions is that at the time of division all the material parts have been duplicated autosynthetically. Therefore, the augmentation of individual enzymes must be at least fairly closely linked with their functioning, and it is a

reasonable working hypothesis to express the rate of increase of each individual one by a differential equation for its autosynthesis. If this is done, then, as will be shown in § 2, automatic response can be accounted for.

It can, however, be said reasonably enough that the material to which this type of equation is applied is not very appropriately called 'enzyme', since isolated enzymes will in fact function without autosynthesis (Medawar 1947). In § 4, therefore, a simple enzyme combination is considered, in which each of two enzymes provides by its functioning the intermediates necessary for the synthesis of the other. Each is found to increase in amount according to an equation identical with that expressing its own autosynthesis. This simple result can probably be generalized to the complex interlocking systems of real cells, and strengthens the case for regarding enzymes as virtually self-reproducing under the conditions of cell growth.

These considerations are based upon the idea that adaptation involves an adjustment of enzyme proportions. The relation between the change of proportions of existing enzymes and the appearance of new ones must therefore be clarified.

The first and simplest approach is to regard modifications in enzyme systems as largely due to combinatory changes in accordance with a view which may be stated as follows.

During long-continued growth, enzymes for which there were no function would be largely eliminated as a result of the relative increase of the others.

The multitudinous enzymatic properties of a cell which are brought into being by adaptive response probably depend upon varying combinations of simple unit processes, their specificity residing largely in their mode of combination, that is, in the order of the unit steps If these individual steps are of a simple and general character (as they could be, for example, with free radical and chain mechanisms), the non-elimination of the relevant enzyme material can be accounted for.

In order to grow in changed conditions a cell may need to use a different combination of its unit processes, in a changed order and in changed proportions.

In the following sections adaptation will first be considered from this purely quantitative point of view, changed proportions being dealt with in § 2 and competing combinations in § 3. In § 5 the relation between the quantitative view and the idea of qualitative changes in the texture of the enzyme material itself will be considered

2. Change of enzyme proportions leading to optimum growth rate

The general ideas underlying this treatment have been outlined elsewhere (Hinshelwood 1946) and will only be indicated briefly. They are here thrown into the form most convenient for our present purpose

Suppose that there are consecutive processes in which cell components ('enzymes') reproduce themselves, the first of the sequence providing the substrate for the second and so on. The first occurs according to the equation

$$dx_1/dt = k_1 x_1,\qquad\qquad . (1)$$

where x_1 is the total amount of 'enzyme 1' in all the bacterial material.

The second occurs according to the equation

$$dx_2/dt = k_2 c_1 x_2, \qquad (2)$$

where c_1 is the concentration of the active intermediate formed by enzyme 1, and is given by the equation

$$n dc_1/dt = \alpha_1 k_1 x_1 - K c_1 n - \alpha_2 k_2 c_1 x_2 = 0. \qquad (3)$$

Equation (3) expresses the balance between formation and loss of the intermediate; the first term gives formation by enzyme 1, the third consumption by enzyme 2 and the second loss by diffusion, which is proportional to the total wall area and in turn to n the total number of cells among which the masses x_1 and x_2 are distributed. α_1 and α_2 are factors expressing the yields of intermediate from enzyme and of enzyme from intermediate respectively.

If the division of the cell waits on the attainment of a critical amount of enzyme 2,

$$n = \beta x_2, \qquad (4)$$

where β is a constant.

The overall growth-rate constant will be given by

$$k = (1/n) \, dn/dt = (1/x_2) \, dx_2/dt. \qquad (5)$$

From equations (1) to (4)

$$\frac{x_1 - (x_1)_0}{x_2 - (x_2)_0} = \frac{\beta K + \alpha_2 k_2}{\alpha_1 k_2} = \rho, \qquad \bullet \qquad (6)$$

where $(x_1)_0$ and $(x_2)_0$ are the amounts at time zero.

It follows also that

$$k = (1/x_2) \, dx_2/dt = k_2 c_1 = (k_1/\rho)(x_1/x_2) \qquad (7)$$

When the culture is stabilized, that is, when it has been growing for a long time in a constant medium, x_1 and x_2 are very large compared with $(x_1)_0$ and $(x_2)_0$, so that

$$(x_1/x_2)_{\text{equil.}} = \rho,$$

and therefore

$$k_{\text{equil.}} = (k_1/\rho)\rho = k_1.$$

Now suppose an inoculum to be transferred to a completely new medium. By the principle formulated in the last section we may suppose that substantially the same enzymes are used in the new sequence, but in a different order and in quite different proportions, e g. a reducing enzyme which played a comparatively minor role in the old scheme may be involved in a key step in the new and so on. The parts played by enzymes 1 and 2 in the old sequence are now taken over by j and k, i e. these latter become 1 and 2 of the new sequence.

Thus

$$(x_1/x_2)_{\substack{\text{initial} \\ \text{new}}} = (x_j/x_k)_{\substack{\text{equil.} \\ \text{old}}}.$$

From (7)

$$k'_{\text{initial}} = (x_j/x_k)_{\substack{\text{equil.} \\ \text{old}}} . (k'_1/\rho'),$$

where the letters with dashes refer to the new growth sequence. Now it may well happen that $(x_j/x_k)_{\text{equil.}}$ of the old sequence is quite small, in which case growth in

the new medium will initially be very slow. But as it proceeds, $(x_j/x_k)_{\text{equil. old}}$ gradually tends to the value $(x_1/x_2)_{\text{equil. new}} = \rho'$, so that by (8) $k'_{\text{equil.}} = k'_1$, i.e. the growth-rate constant, starting from a very low value, rises eventually to the optimum value k'_1 which the mechanism allows.

Adjustments of this kind will only occur at rates comparable with the growth rate itself and will only be completed in so far as the bacterial substance formed under the new conditions outweighs that originally present.

3. COMPETING REACTION SEQUENCES

It is now necessary to investigate the question as to which route will be followed when several combinations of elementary enzymatic steps can lead to the final result. The existence of these alternative combinations is implicit in the propositions enumerated in the introduction For example, a substance might be deaminated and then involved in a condensation reaction, or these two steps might occur in the reverse order, and so on. The general treatment of the problem would be very complex, but the following very simple calculation probably contains the essential result.

Suppose enzyme 1 undergoes its autosynthetic expansion coupled with a step in the series of growth reactions and yields an intermediate at concentration c_1, used by enzyme 2 in some process (also coupled with autosynthesis). With assumptions similar to those of the last section we should have

$$dx_1/dt = k_1 x_1, \quad dx_2/dt = k_2 c_1 x_2, \quad n\,dc_1/dt = \alpha k_1 x_1 - K n c_1 = 0.$$

Here, to reduce the number of symbols, the utilization of the intermediate is taken to be small compared with its loss by diffusion. This makes no difference at all in principle, as may be seen by reference to the previous section, where the omission of the last term in equation (3) would leave all subsequent steps unchanged. We shall suppose that the order of operation can also be reversed, enzyme 2 playing the part of the initiator and yielding a different intermediate at concentration c_2, used by enzyme 1, so that

$$dx_2/dt = k'_2 x_2, \quad dx_1/dt = k'_1 c_2 x_1, \quad n\,dc_2/dt = \alpha' k'_2 x_2 - K' n c_2 = 0.$$

If both routes are possible, we should have the system of equations

$$dx_1/dt = (k_1 + k'_1 c_2)\, x_1, \tag{1}$$

$$dx_2/dt = (k'_2 + k_2 c_1)\, x_2, \tag{2}$$

where

$$c_1 = (\alpha k_1/Kn)\, x_1, \tag{3}$$

and

$$c_2 = (\alpha' k'_2/K'n)\, x_2 \tag{4}$$

Case 1. Division is determined by x_2 so that $n = \beta x_2$. The solution of the equations is then

$$x_1 = (x_1)_0\, e^{\chi t},$$

where

$$\chi = k_1 + \alpha' k'_1 k'_2/K'\beta$$

and

$$x_2 = \frac{\alpha k_1 k_2}{K\beta(\chi - k'_2)}\{(x_1)_0\, e^{\chi t} - (x_1)_0\, e^{k'_2 t}\} + (x_2)_0\, e^{k'_2 t}. \tag{5}$$

If $\chi > k_2'$, x_2/x_1 tends to the constant limit $\alpha k_1 k_2 / K\beta(\chi - k_2')$ as growth proceeds. As this is approached c_1 tends to the value $\alpha k_1 K\beta(\chi - k_2')/K\beta\alpha k_1 k_2$ so that

$$dx_2/dt = k_2' x_2 + (\chi - k_2') x_2 = \chi x_2,$$

and both enzymes grow with the same specific rate χ.

If χ were less than k_2', x_1/x_2 would tend to zero as growth proceeded. Before this limit was reached the division condition would have to change to $n = \beta' x_1$. Thus the above represents the only stable condition.

Case 2. Division is determined by x_1, so that $n = \beta' x_1$. In this case the solution is

$$x_2 = (x_2)_0 e^{\phi t},$$

where

$$\phi = k_2' + \alpha k_1 k_2 / K\beta'$$

and

$$x_1 = \frac{\alpha' k_1' k_2'}{K'\beta'(\phi - k_1)} \{(x_2)_0 e^{\phi t} - (x_2)_0 e^{k_1 t}\} + (x_1)_0 e^{k_1 t}. \tag{6}$$

Here the limiting value of x_1/x_2 is $\alpha' k_1' k_2'/K'\beta'(\phi - k_1)$ so long as $\phi > k_1$. Otherwise x_2/x_1 would tend to zero and the division condition would have to change to case 1. With the above limit a value of c_2 is established which ensures that both enzymes grow with the specific rate constant ϕ.

We thus have the two stable conditions

$$\chi = k_1 + \alpha' k_1' k_2'/\beta K' > k_2' \quad \text{Rate constant } \chi$$

and

$$\phi = k_2' + \alpha k_1 k_2/\beta' K > k_1 \quad \text{Rate constant } \phi.$$

Suppose, for example, in case 2 that we start with $(x_1)_0$ far greater in relation to $(x_2)_0$ than corresponds to the stable ratio. Initially the most important term in equation (6) will be $(x_1)_0 e^{k_1 t}$, because $(x_1)_0$ is small, and the rate constant will initially have the value k_1. As growth proceeds this term will be superseded by that involving $e^{\phi t}$, which after a time will be the only important one. Thus the rate constant will undergo a transition from k_1 to ϕ, giving rise to the appearance of a segmented growth curve. The combination giving the most rapid overall growth rate establishes itself.

4. Indirect Coupling of Synthesis and Function

In the foregoing discussions we have employed equations which express the direct autosynthesis of the enzyme material as it exerts its function in the cell. This matter is worthy of more detailed consideration

The direct coupling of autosynthesis and function might well be considered in general to present some difficulty. The following calculation indicates, however, that an indirect coupling could have a very similar effect

Suppose enzyme 1 grows by use of a substrate derived from another enzyme j, and whose concentration is c_j. Let its formation be given by

$$dx_1/dt = \alpha_1 k_1 c_j,$$

that is, a non-autocatalytic process. The intermediate is formed from enzyme j acting on other medium constituents. In this case the equations are more easily

handled if we may neglect losses by diffusion, a condition, incidentally, which is likely to be approximately fulfilled in certain real cases. We write

$$dc_j/dt = k_j' x_j - k_1 c_j = 0.$$

Now enzyme j is in a similar case and we have

$$dx_j/dt = \alpha_j k_j c_k, \quad dc_k/dt = k_k' x_k - k_j c_k = 0,$$

where c_k is the concentration of an intermediate derived from another enzyme k. There can be similar cross-linking of various other enzymes and intermediates. The essentials of the problem are preserved if we take x_j to refer to enzyme 2, and the enzyme k which feeds it to be enzyme 1, so that

$$dx_1/dt = \alpha_1 k_1 c_2, \quad dc_1/dt = k_1' x_1 - k_2 c_1 = 0,$$
$$dx_2/dt = \alpha_2 k_2 c_1, \quad dc_2/dt = k_2' x_2 - k_1 c_2 = 0.$$

From these it follows that

$$dx_1/dt = A x_2 \quad \text{and} \quad dx_2/dt = B x_1,$$

where A and B are constants.

The solution of these equations is

$$x_1 = \tfrac{1}{2}\{(x_1)_0 + (A/C)(x_2)_0\} e^{Ct} + \tfrac{1}{2}\{(x_1)_0 - (A/C)(x_2)_0\} e^{-Ct},$$
$$x_2 = \tfrac{1}{2}(C/A)\{(x_1)_0 + (A/C)(x_2)_0\} e^{Ct} - \tfrac{1}{2}(C/A)\{(x_1)_0 - (A/C)(x_2)_0\} e^{-Ct},$$

where $C^2 = AB$. When t becomes large the ratio x_1/x_2 tends to the constant value $A/C = C/B$, the terms in e^{-Ct} becoming negligible.

Now $$(1/x_1) \, dx_1/dt = A x_2/x_1 \quad \text{and} \quad (1/x_2) \, dx_2/dt = B x_1/x_2.$$

When t is large, both these attain to the constant value C, i e. when t is small the rates approach

$$1/(x_2)_0 \, dx_2/dt = B(x_1)_0/(x_2)_0 \quad \text{and} \quad 1/(x_1)_0 \, dx_1/dt = A(x_2)_0/(x_1)_0.$$

If $(x_1)_0$ has a very small value the rate of growth of x_2 is so small that the division of the cell is delayed; if $(x_2)_0$ is very small then the growth of x_1 is delayed.

The above calculation, although much oversimplified, serves the purpose of showing that there is no inconsistency between the assumption of autosynthetic behaviour in an actual cell, and the recognition of the fact that isolated enzymes may function without growth.

5. QUALITATIVE AND QUANTITATIVE ENZYME CHANGES

Autosynthesis of enzymes, whether direct or indirect, can only occur at specialized sites (the need for an energetic coupling of reactions limiting to a considerable extent the degree of indirectness possible). At the sites where the utilization of substrate occurs it seems almost certain that suitable spacings and foldings of the protein base material of the cell must exist. Moreover, when autosynthesis occurs on these bases, the new material will certainly be laid down in such a way as to conform to the

pattern of the old. The spacings and foldings may well show a statistical variation from one element of cell volume to the next, and some of them may be more suitable than others for dealing with a new substrate.

Thus we might modify or reinterpret the idea that there exist small amounts of all the enzymes necessary for the unit processes of the cell by supposing that certain kinds of spacing, susceptible of continuous variation (by changes of folding for example), happen to be adjusted better than others to the new chemical function.

These favoured sites would (since, as stated, the existing material guides the deposition of the new) expand during growth with utilization of the new substrate. Thus we should have a quantitative expansion, as envisaged in the hypotheses of the earlier sections, but one due to what might be called internal selection.

This point of view would reconcile hypotheses based upon 'expansion of enzymes' with those based upon 'change of texture', and also, in some degree, hypotheses of direct adaptation with those of selection

6. Reversibility of adaptive changes

The equations of earlier sections imply easy reversibility of adaptive changes under conditions where the newly developed function is no longer in use and growth occurs in the original medium. In fact, however, adaptation to a new medium is not readily lost, and, within the framework of the hypothesis used, we must suppose that the modified enzyme systems continue to be kept in some kind of employment when the cells are returned to their original medium The complete discussion of this question will not be entered into here, but one relevant factor is the following. Let A be the normal carbon source, e.g glucose, and B be an alternative to which adaptation occurs. The sequence of reactions can be schematized thus:

$$\begin{array}{c} A \\ \diagdown \\ C - D \ ..., \\ \diagup \\ B \end{array}$$
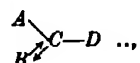
where C represents the stage at which the two alternative reaction routes join. Since most enzymes are reversible, development of the route $B \to C$ will also develop that of $C \to B$. Thus, when cells adapted to utilize B are grown on A, a certain amount of the intermediate C will be converted into B and vice versa. The enzyme dealing with B will therefore continue to function and its synthesis can also continue. Another factor of possible importance is that B may be only a modified form of A, capable of dealing with A as well as the unmodified form. This idea has been discussed briefly elsewhere (Postgate & Hinshelwood 1946).

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The thickness of the myelin sheaths of normal and regenerating peripheral nerve fibres

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An investigation has been made of the relation between axon diameter, fibre diameter, and myelin sheath thickness in myelinated nerve fibres from the peroneal nerve of the rabbit. Fibres were measured· (a) in the normal nerve, and (b) during regeneration following nerve crushing.

In the normal nerve, fibres ranging in diameter from 1 to 20μ were present. These had axons with diameters from 0.5μ in the smallest, to about 15μ in the largest fibres. The 0.5μ axons had myelin sheaths about 0.5μ thick, while the largest axons had sheaths about 2.5μ thick. Between these extremes the curve relating sheath thickness to axon diameter was at first steep, and then more gradual in slope.

In regenerating nerve fibres 15 mm. proximal to the site of lesion the *axons* decline steadily in diameter during the first period of regeneration (100 days) Thereafter they increase in size, although even after 300 days the largest axons have not regained their normal diameter The axons, 10 mm. below the lesion, extremely thin at their first appearance, increase steadily in diameter, until, 300 days after injury they equal in diameter the axons of the proximal stump.

The decrease in diameter of axons proximally is accompanied by an absolute increase in their myelin sheath thickness This takes place first of all without any alteration in the total diameter of the fibres concerned, the increase in thickness of the myelin compensating the decrease in axon diameter Between 60 and 100 days after injury, however, continuing axon decrease is accompanied by a decrease in the total diameter of the fibres. Between 100 and 200 days an increase in total fibre diameter parallels the increase in axon diameter, the myelin sheaths remaining abnormally thick. Between 200 and 300 days after injury the continued increase in axon diameter causes a diminution in myelin sheath thickness. Even at 300 days after injury, however, the sheaths are still thicker than in normal nerve.

In the distal stump the myelin sheaths, when first developed, are thicker than those of fibres of corresponding diameter in normal nerve Subsequently they grow still thicker, reaching their maximum thickness about 200 days after nerve injury Between 200 and 300 days a slight diminution in sheath thickness occurs since the axons continue to increase while the total diameter remains the same However, even at 300 days the sheaths are still thicker than normal.

The bearing of these results on the nature of the forces maintaining the structure of nerve fibres is discussed.

INTRODUCTION

All nerve fibres are surrounded by a sheath which contains radially orientated lipide molecules and tangentially arranged protein molecules, the two components forming a concentrically layered structure, indeed, the presence of a sheath of this type seems essential for the propagation of the nervous impulse (Schmitt & Bear 1939). In those mammalian nerve fibres which exceed 1 to 2μ in diameter there is enough lipide present to cause the sheath to blacken in osmium tetroxide solutions, and it is such fibres which are conventionally termed 'myelinated' There is no doubt that it is the possession of a relatively thick sheath containing a high proportion of lipide which gives these fibres their high rate of conduction compared

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with 'non-myelinated' fibres of similar diameter, although it is not known which is the significant variable; sheath thickness, or the proportion of lipide in the sheath (see Pumphrey & Young 1938; Schmitt & Bear 1939; Taylor 1942; Taylor & Werndle 1943, Sanders & Whitteridge 1946).

In mammalian mixed somatic nerves the 'myelinated' fibres range in size from 20μ to about 1μ in stained preparations (Duncan 1934), and their sheaths vary in thickness and composition (Schmitt & Bear 1939). When such nerves are interrupted, both the myelin sheaths and the axons of the peripheral stump disintegrate and are removed, reinnervation being accomplished by the outgrowth of fresh axons from the central stump. Later (Gutmann & Sanders 1943) it was shown that regeneration tends to restore a frequency distribution of fibre sizes in the nerve similar to that which was originally present, although complete reconstitution of the nerve does not occur except after simple localized crushing. Hence, as the degree of myelination of nerve fibres also has functional importance, we may suppose that regeneration will tend to restore fibres whose sheaths have the appropriate thickness and ultrastructure. However, while it is well known that regenerating 'myelinated' nerve fibres are at first thin and 'non-myelinated', and only later acquire osmium tetroxide-reducing sheaths complete with Ranvier nodes (Hentowa 1934, Young 1942), the later phases of remyelination have been little studied. We do not know what controls the thickening of the concentrically layered lipo-protein sheath initially formed around a nerve fibre, although the size of the axon which the sheath surrounds is possibly a factor (see Duncan 1934, Schmitt & Bear 1939).

It was with this possibility in view that the experiments described in the present paper were undertaken. The paper reports a series of measurements of the sheath thickness and axon diameter of 'myelinated' nerve fibres at different times after the interruption of the nerve by crushing. Besides the regenerating fibres in the peripheral stump, their parent fibres in the central stump were also measured. Greenman (1913) and Gutmann & Sanders (1943) found that during regeneration the myelinated fibres of the central stump underwent a reduction in total diameter. When the nerve had been crushed this decrease was progressive for about 130 days after injury, and thereafter the diameter gradually increased to the normal value. The decrease in total diameter of the central stump fibres was accompanied by a corresponding increase in diameter of the new fibres in the periphery, and it was suggested that part, at least, of this increase was due to a flowing down of axoplasm from parent fibres in the central stump. The suggestion was based, however, on the results of measuring *total diameters* (i.e. axon + myelin sheath) of a large number of fibres, and has been criticized by Hammond & Hinsey (1945) who suggested that an apparent diminution in the mean fibre diameter of the central stump could be produced by the presence of small regenerated fibres which had doubled back at the lesion and grown up the central stump. Although such a doubling back rarely occurs following a crush lesion it was thought desirable to undertake a revaluation of the data in terms of *axon diameters*, which would provide a much better measure of axoplasmic outflow For example, a transient demyelination of the central stump fibres could produce an apparent shrinkage in total diameter, without any change

in the axoplasm content. Measurements of fibres central to the lesion were therefore undertaken to discover whether the shrinkage observed during regeneration in these fibres was indeed due to a loss of axoplasm, or to some other process.

METHODS

All the measurements were made upon fixed and stained myelinated nerve fibres. Pieces of nerve were removed from four rabbits whose peroneal nerves had been interrupted by firm localized crushing with smooth-tipped forceps 60, 100, 200, and 300 days previously. The lesion was in each case made approximately 80 mm. above the level of entry of the nerve into the most proximal of the muscles it supplies and approximately 100 mm below the spinal cord, and at biopsy pieces were taken (*a*) from the central stump 15 mm. above the lesion, and (*b*) from the peripheral stump 10 mm distal to the site of crushing. Specimens of normal nerve at similar levels were also taken from five previously unoperated animals

The nerves, lightly stretched on pieces of library card, were fixed in Flemming's osmo-chrome-acetic mixture. They were sectioned transversely at 4 to 5μ and stained according to the modified Weigert technique described by Gutmann & Sanders (1943) Parts of sections were selected in which there was the least distortion of the myelin sheaths, and photographs made of each nerve at a magnification of 1200 diameters by direct projection through the microscope on to bromide paper.
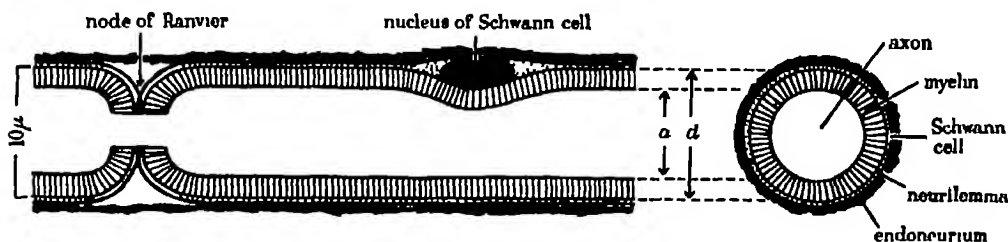
FIGURE 1 Diagram of the appearance of a stained myelinated nerve fibre to show the quantities measured $a =$ axon diameter, $d =$ total diameter. The proportions of axon and sheath in the figure are roughly those of a 10μ fibre

Using the photographs obtained in this way, measurements were made with a travelling microscope of the inside (*a*, figure 1) and outside (*d*, figure 1) diameters of selected fibres. The inside and outside diameters were measured twice for each fibre, the two measurements being made in directions at right angles to one another The means of the two values of *a* and *d* obtained in this way were recorded as the axon and total diameter of the fibre in question. In the case of normal nerve, central stumps, and the 200- and 300-day regenerates, where a considerable range of fibre sizes was present, 160 to 200 fibres were measured in each nerve In the two early regenerating stumps (60 and 100 days), 100 and 133 fibres were measured. In every case the fibres were chosen so that the population of measurements lay evenly scattered over the range of fibre sizes.

TREATMENT OF DATA

From the data obtained in this way the following quantities were calculated for each fibre. (1) The relation between the cross-sectional areas of the myelin sheath and the axon. According to figure 1, the cross-sectional area of the myelin sheath $= \pi(d^2 - a^2)/4$, and that of the axon $= \pi(a^2/4)$. Thus the 'area ratio' (i.e. sheath area divided by axon area) $= (d^2 - a^2)/a^2$. (2) The ratio g (see Schmitt & Bear 1937), which is the ratio of axon diameter to total diameter (a/d, figure 1). Graphs were then plotted: (a) of the ratio g against total diameter; (b) of the *area ratio* against axon diameter; and (c) of $2 \times$ the myelin sheath thickness ($d - a$, figure 1) against axon diameter.

As a result of the various sources of non-systematic error discussed on pp. 329 to 330, the points representing individual fibres in these graphs are more or less evenly scattered about smooth curves which represent the relationship between the quantities plotted. In the case of graphs of g and the *area ratio*, such curves were drawn by eye alone. However, in the case of graphs of $2 \times$ myelin thickness against axon diameter, which were more susceptible to statistical treatment, an attempt was made to fit curves to the data by a statistical method.

In studies on relative growth it has been found that the formula of simple allometry (i.e. organ size $= K \times$ body size α) has a wide application. When such data are plotted on double logarithmic coordinates, they lie evenly about a straight line whose slope and intercept on the Y-axis can be found by the classical methods of linear regression and the formula of the relationship determined. Moreover, a test of goodness of fit can be employed to discover whether the straight line so obtained is indeed a satisfactory fit to the data. In the present experiments it was thought probable that graphs of myelin sheath thickness against axon diameter could be represented by straight lines when plotted on double logarithmic coordinates. In the case of all nerves, therefore, in addition to curves with linear coordinates, double logarithmic plots were made of sheath thickness against axon diameter. From these double logarithmic graphs the curves giving the best fit to the linear graphs were calculated in the following way. First of all, the regression line to fit the whole data was calculated, and to this line a statistical test of goodness of fit was applied. The data were set out in the form of a correlation table and the 'variance within arrays', and the 'residual' variance, due to the deviations from the regression calculated, each with its appropriate number of degrees of freedom. From these the *variance ratio* could be obtained. By consulting the table of this ratio given by Fisher & Yates (1943) it was then possible to ascertain whether the graph showed a significant deviation from linearity, a probability of less than 0.05 was regarded as indicating that a straight line was inadequate to describe the given data. In all cases it was found that a single straight line, by this criterion, did not give a good fit to any of the double logarithmic graphs. Next, an attempt was made to fit two straight lines, and so on until a significant fit with multiple straight lines was obtained. As most of the double logarithmic graphs showed an obvious kink in one or more places, this proved a practicable method. It is realized, of course, that an even better fit would be obtained by using a curve, but the multiple straight lines are a valuable

approximation until more precise methods are required. From the multiple straight lines fitted to the double logarithmic plot the curves fitting the linear graphs were found by replotting the lines on the original linear coordinates.

ACCURACY OF RESULTS

The accuracy of the estimates given in different cases of sheath thickness, axon diameter, and the relation between them, is affected by errors which may be classified as follows:

(1) *Errors of the preparation*

All the measurements were made upon fixed and stained nerve fibres. It is generally assumed that the processes of fixation, embedding, and staining cause nerve fibres to shrink, but the exact extent of the error introduced by this factor is not known. On *a priori* grounds the processes of preparation can affect the measurements in one of four ways· (*a*) they can cause a regular, proportional shrinkage of both axon and myelin, altering the absolute values of axon diameter and sheath thickness, without changing the relation between them, (*b*) a regular, but differential shrinkage of axon and/or myelin can occur, which will alter both the absolute values of axon diameter and/or myelin thickness and the relation between them; (*c*) shrinkage can affect fibres of different sizes to a different extent, so that the curve of myelin thickness against axon diameter is distorted, or (*d*) there can be an irregular shrinkage of axon and/or myelin, with the consequent production of artefacts.

Few authors have considered all these possible types of shrinkage, most being concerned only with shrinkage in total diameter. Sherrington (1894) who compared fresh fibres teased in physiological saline with fibres fixed in osmium tetroxide and teased in glycerine, found that osmium tetroxide fixation caused no change in fibre diameter. Similarly Lapicque & Desoille (1927) compared the dimensions of fresh teased fibres with paraffin cross-sections of osmicated fibres and concluded that no shrinkage occurred. Duncan (1934) also states that there is no alteration of fibre diameter as a result of fixation in ¼ to 2 % osmic acid, dehydration, and subsequent embedding in paraffin.*

Evidence to the contrary is given by Arnell (1936) who compared the cross-sectional areas of axon and myelin sheath in fresh fibres, in formol-fixed frozen-sectioned fibres, and formol-fixed paraffin-sectioned fibres stained with methylene or by a silver method. He found that formol fixation caused shrinkage, and paraffin embedding still further shrinkage, in both axon and myelin. However, he does not state whether both shrink to the same extent. Moreover, the actual values of his measurements show considerable variation, and as he measured only a small number of fibres, his results are not conclusive. In addition, since he used only formol as a fixative, a substance which does not render the lipoids of the myelin sheath resistant to subsequent extraction by the alcohol used for dehydration, the value of his results is questionable. Taylor (1942), who measured fresh teased fibres

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* Donaldson & Hoke (1905) found shrinkages in mammalian nerve of only 0 3 %, but as they measured the dimensions of whole nerves rather than those of fibres, their results are not strictly comparable with those of other workers.

in polarized light, and compared his results with those of authors who used fixed and stained material, claimed that fixation and staining caused the axon to shrink more than the myelin.

The clearest evidence for shrinkage is that of Hursh (1939). This author measured nerve fibres teased out from a spinal root bundle which had been fixed in OsO_4, individual fibres were measured before and after dehydration. He found that the fibres underwent a shrinkage of $10.1 \pm 0.16\%$, but that there was no correlation between the percentage of shrinkage and the size of the fibre.

From the above evidence it may be concluded that shrinkage of fibres in total diameter does occur during the processes of microscopical preparation, the greater part of the shrinkage probably taking place during dehydration and paraffin embedding rather than during fixation. Moreover dehydration and embedding can often cause the axon to shrink more than the myelin. In silver-stained preparations, or in counter-stained myelin sheath preparations, an empty space can generally be seen between the axon and the inner border of the myelin sheath. However, in experiments where axon and total diameters are measured, the axon diameter is generally taken as equal to the distance between the inner borders of the myelin sheath, on opposite sides of the fibre. The problem of the differential shrinkage of axon and myelin during microscopical preparation thus resolves itself into the question whether the myelin sheath decreases in thickness to relatively the same extent as the whole fibre diminishes in diameter.

An attempt was made to answer this question in the following way. pieces of normal nerve were teased rapidly in Ringer's solution, and photographs of individual fibres taken at a magnification of $\times 1200$ within 15 min. of taking the pieces. These photographs were taken with a petrographic microscope, with the revolving stage adjusted to give maximum brightness to the myelin sheath. Fifty-six undistorted fibres were selected and the inner and outer diameters of their myelin sheaths measured on the photographs by means of the travelling microscope. Five such pairs of measurements were made on each fibre, and the means of the five pairs taken as the value for the fibre concerned. These measurements were compared in the following manner with those made on 200 normal fibres fixed and stained in the course of the present work. both fixed and unfixed fibres were divided into groups differing by steps of 1μ axon diameter, and for each the mean axon diameter, total diameter, and myelin sheath thickness were found. Graphs were then made for both fresh and fixed fibres of (a) sheath thickness against axon diameter, and (b) total diameter against axon diameter, on a double logarithmic scale. In all four graphs the points plotted were scattered about a straight line, which, by the test of goodness of fit outlined above (see p. 326) was a satisfactory fit to the data. The sheath thickness/axon diameter and total diameter/axon diameter regressions were then compared by the test described by Reeve (1940). In this test the residual variances (i.e. variance due to deviations from regression) after fitting (a) a single regression line, (b) two parallel lines, (c) two separate lines to the data for fresh and fixed fibres were used to calculate variances, due to (d) differences in slope, and (e) differences in position, between the lines for fresh and fixed fibres. By using the variance ratio the significance of the latter differences could be

determined. In the case of both sheath thickness and total diameter the regression line on axon diameter for fresh fibres did not differ significantly in slope from that for fixed fibres This indicates that there is no differential effect of shrinkage 6n fibres of different diameters. In both cases, however, the regression lines for fresh differed significantly in position from those for fixed fibres, indicating that shrinkage does take place. In the case of the sheath thickness/axon diameter regression fresh fibres had sheaths which were $1 \cdot 08 \pm 0 \cdot 02$ times as thick as those of fixed fibres: in the case of total diameter, fresh fibres had diameters which were $1 \cdot 06 \pm 0 \cdot 017$ times those of fixed fibres. These two values are not significantly different from one another, which indicates that the myelin sheaths decrease in thickness in the same proportion as the total diameter. These results indicate that a shrinkage of 6 to 8 % is brought about in nerve fibres by fixation and embedding and that this shrinkage is of the first type described above (p. 327)—namely a regular, proportional decrease in axon and total diameter which alters the absolute values of the measurements, but does not affect the relation between them.

That this is not the only type of shrinkage which can take place is shown by the frequency with which artefacts are found in stained nerves. What the above experiment indicates, however, is that, provided the fixed fibres selected are round and free from artefact, the measurements will differ from those of fresh fibres by a predictable amount

In the present work only those fibres were selected for measurement which fulfilled the above criteria, i e were approximately circular and free from artefact, the state of each fibre in the photograph being controlled before measurement by reference to the actual preparation Furthermore, to make the results from different nerves as nearly comparable as possible, all the pieces were treated similarly during fixation, embedding, and staining, being left for equal times in the various solutions.

(2) *Errors introduced by photography*

The use of photographic methods introduces three possible sources of error (*a*) photographs taken upon different occasions may vary slightly in magnification; this error was reduced as far as possible by carefully checking the magnification with the same micrometer slide before each photograph was taken, (*b*) slight variations in focus or exposure may blur the outlines of the myelin sheaths and so give rise to errors of measurement, this was controlled by very careful focusing under a magnifier, and by making measurements only on bright, correctly exposed photographs, (*c*) the dimensions of the photographic images of fibres may be altered by slight movements of the photographic emulsion during the process of making a negative and printing from it; this error was unlikely to be great as the photographs were made directly as negatives on bromide paper An additional advantage of this method was that it proved easier to measure to the borders of a white object on a black ground than vice versa.

(3) *Errors of measurement*

Two sources of error enter in here: (*a*) It has been suggested by Taylor (1942) that when measuring stained sections of myelin sheath material it is easy to include some of the Schwann sheath or the endoneurium in an estimate of myelin sheath

thickness. This possibility was checked as follows. Two adjacent sections of a piece of normal nerve were stained as above (p. 325). One of them was then counter-stained with light green, which shows up the endoneural sheaths. Photographs of both sections were then taken, and the thickness of the myelin sheath measured in the same 50 fibres in each case. The difference between each of the 50 pairs of measurements was then calculated. The mean difference between the 50 pairs of measurements was 0.162μ; $t = 0.53$ for 49 degrees of freedom, which from the table of t given by Fisher & Yates (1943) shows that differences of this amount occur in over 60 % of cases as a result of random sampling alone. No systematic error, therefore, is introduced by measuring the myelin sheath in sections stained for the myelin alone. (b) All the measurements were made by means of a travelling micro-scope. The error of measuring with this instrument is unlikely to be systematic, but it was estimated as follows. Each of 10 fibres, ranging in diameter from 2μ to 20μ, was measured ten times, the individual measurements being made in a random order. From the 100 values so obtained the total sum of squares $\Sigma(d - \bar{d})^2$ was calculated, corresponding to 99 degrees of freedom   From this a component corresponding to the 'variance between fibres' with 9 degrees of freedom was subtracted, leaving a sum of squares with 90 degrees of freedom which could be used as an estimate of error. The data of this analysis of variance are set out in table 1. The root mean square had a value of $\pm 0.18\mu$, which corresponds to about ± 9.0 % of the diameter for a 2μ fibre and ± 0.90 % for a 20μ fibre.

TABLE 1. ANALYSIS OF VARIANCE OF ERRORS OF MEASUREMENT

item	degrees of freedom	sums of squares	mean square
difference between fibres	9	1234 41	—
error	90	4·06	0 0451
total	99	1238·47	—

root error mean sq $= \pm 0\ 21276$ mm. which, at a magnification of $\times 1200 = \pm 0.18\mu$.

(4) *Age, size, sex and race of animals*

No attempt was made to control these factors. Since only slight differences exist in the maximum size of nerve fibres in mammals as diverse in size and genetic constitution as rat, cat and cow (Duncan 1934) it it unlikely to have introduced any appreciable error. As only healthy, adult animals were used, alterations in fibre diameter due to pathological changes can also be discounted.

Total effect of errors

From consideration of all these sources of error it is apparent that the only serious systematic error in the methods used is that due to the decrease in axon and myelin dimensions produced by fixation, embedding, and staining. However, as neither the relation between axon and myelin dimensions, or the dimensions of fibres of different sizes, is affected differentially by this source of error, the shrinkage can be corrected for by multiplying all the measurements by a factor of 1·07. This has not been done throughout the text, and unless otherwise stated, all the measurements

quoted refer to fixed and stained fibres. As all the pieces were treated comparably, this source of error does not affect comparisons made between the different nerves. All the other errors are non-systematic.

THE RELATIONSHIP BETWEEN THE DIMENSIONS OF MYELIN SHEATH AND AXON IN NORMAL NERVES

Previous authors have made use of two quantities to describe the relation between the dimensions of the axon and the myelin sheath of nerve fibres. These are (1) the ratio between the cross-sectional areas of myelin and axon (Donaldson & Hoke 1905, Arnell 1936), and (2) the ratio g (Schmitt & Bear 1937 *et seq.*; Taylor 1940 *et seq*) (see also p. 326).

Donaldson & Hoke (1905) were the first to measure the axon and total diameters of vertebrate nerve fibres, and to attempt to find a relation between them. They examined 45 pieces of nerve from 27 species, measuring in all 1540 fibres. From their original measurements they calculated the ratio between the cross-sectional area of the myelin sheath and that of the axon, and in every case found that, within the limits of the experimental error, this ratio had a value of 1:1. Greenman (1913, 1916–17), who made direct measurements of the cross-sectional areas by means of a planimeter in photographs of rat fibres, found a similar relation.

In figure 2 the data from 200 normal nerve fibres have been treated in a similar way, the *area ratio* $((d^2 - a^2)/a^2$, see figure 1 and text, p. 326), for each fibre being plotted against its axon diameter Were the *area ratio*, as claimed by Donaldson & Hoke (1905), constant at 1 on all fibres, the values for individual fibres should lie about a straight line parallel to the base line, cutting the Y-axis at 1, as shown by the dotted line in figure 2. From figure 2 it is clear that the relation between the cross-sectional areas of myelin sheath and axon does not have a constant value, but varies with axon diameter, the curve relating the two having the general form of a rectangular hyperbola. For fibres with axon diameters of less than 0.5μ the *area ratio* is as high as 24, though declining rapidly with increasing axon diameter. In the range of axon diameters 1 to 5μ the slope of the curve undergoes a rapid change, the *area ratio* at 5μ having a value of about 1·50. Thereafter the ratio only declines gradually, reaching a final value of about 0 70 at an axon diameter of 14 to 15μ In other words, the smallest fibres have myelin sheaths with a cross-sectional area many times that of the axon. With progressively larger fibres, the proportion of the total cross-section occupied by the myelin declines. This decline is steep at first and then becomes much more gradual, so that the largest fibres have myelin sheaths whose areas are a little less than half that of the entire fibres.

Figure 2 also shows that the only fibres for which Donaldson & Hoke's 1.1 ratio can be said to hold are those which have axons between 8 and 10μ in diameter. Careful inspection of their data shows that most of the fibres measured by these authors had fairly large diameters. With their method of measurement (an eyepiece micrometer at a magnification of 265 to 340 diameters) they may easily have missed the relatively small decline in the *area ratio* which takes place in the tail of the curve; hence their results are not necessarily in conflict with the present ones.

Unfortunately, their data are not presented in a way which makes it possible to discover whether in fact such a decline was present. Similar considerations apply to the data of Greenman (1913, 1916–17) who measured only the 40 largest fibres in each of his nerves.
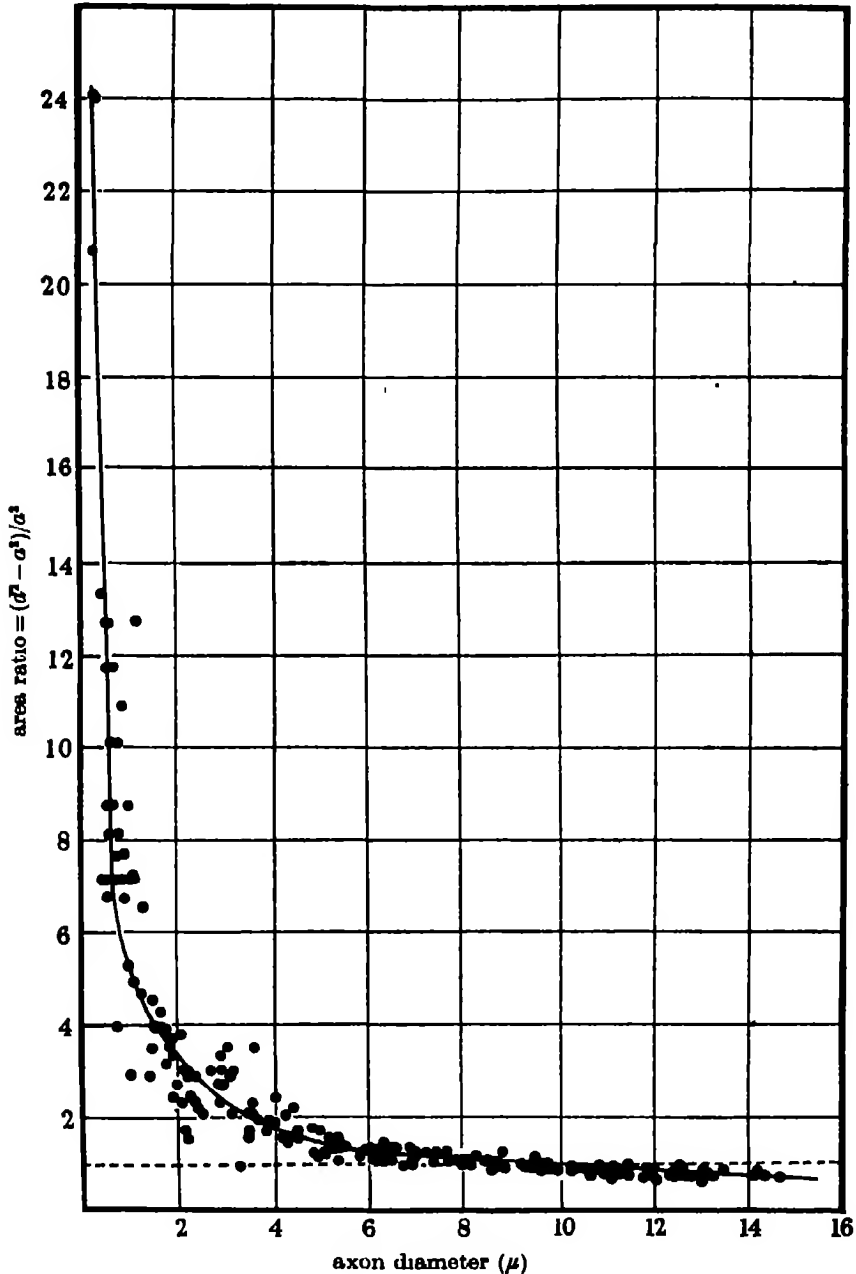

FIGURE 2. Graph of the area-ratio against axon diameter for 200 selected normal myelinated fibres. The curve was drawn by eye.

The results given in figure 2 are also contrary to those of Arnell (1936). This author measured the *area ratio* in fibres from the dorsal and ventral roots of spinal nerves in the dog and man. He divided the fibres into three arbitrary groups—namely (1) those with axons greater than 3.64μ in diameter, (2) those with axons between 1.46 and 3.64μ in diameter, (3) those whose axons had diameters of less than 1.46μ. He measured 20 to 25 fibres in each group, and calculated the mean value of *axon diameter* and *area ratio* for the group. As a result of these measurements he claimed that in all his preparations the largest fibres had relatively thinner myelin sheaths than the others, the middle group (axon diameters 1.46 to 3.64μ) relatively the thickest sheaths. This finding does not agree with the data of figure 2 above. However, Arnell's method of measurement was a crude one. His measurements were made roughly by means of an ocular grid at a magnification of $\times 600$. The smallest division of the grid was equivalent to a distance of 0.82μ on the section, and so his figure for the smallest fibres is unlikely to have been accurate.

Another measure of the relative proportions of axon and myelin in fibres of different diameters is the ratio g, first used by Schmitt & Bear (1937), and subsequently by other authors (Gasser & Grundfest 1939, Taylor 1940, 1941, 1942, Taylor & Werndle 1943). Schmitt & Bear (1937) calculated g for frog fibres, of which the axon and total diameters had been measured in polarized light. They found g to vary in value from 0.48 on fibres of 2.3μ diameter to 0.82 on a 16.4μ fibre. Above 9μ g had a relatively constant value. However, these authors only give data for 29 fibres, and the exact form of the relationship between g and fibre diameter cannot be found from their measurements. Gasser & Grundfest (1939) measured a number of cat saphenous fibres in an osmium-tetroxide fixed preparation, and found that fibres whose total diameter exceeded 8μ had a g value which was relatively constant at 0.69.[*] Below 8μ g became rapidly smaller with decreasing fibre diameter. Taylor (1942), who, like Schmitt & Bear (1937), measured fresh nerve fibres in polarized light, found that in 113 fibres taken from the sciatic and saphenous nerves of the cat, g had values which, when plotted against fibre diameter, lay about a smooth curve, the ratio increasing from a value of 0.30 at a fibre diameter of 2μ to 0.82 at 16μ.

In figure 3 the value of g for the 200 normal rabbit peroneal fibres measured in the present work are shown plotted against total fibre diameter. Taking into account scatter of the points due to the various sources of error, the individual points lie along a curve, g increasing with fibre diameter from a value of about 0.30 at a fibre diameter of 2.5μ to 0.75 at a diameter of 16 to 18μ. The curve is upwardly convex, g at first increasing rapidly with fibre diameter, latterly only showing a gradual increase. Taking into account differences in technique, this curve closely resembles the one given by Taylor (1942). In common with his, these results are at variance with those of Gasser & Grundfest (1939), in that there is no plateau

[*] The value of $g = 0.69$ of Gasser & Grundfest (1939) expresses the same relation between axon and sheath dimensions as Donaldson & Hoke's (1905) 1.1 ratio, for $\dfrac{d^2 - a^2}{a^2} = \dfrac{1}{g^2} - 1$ whence, if $\dfrac{d^2 - a^2}{a^2} = 1$, $g = 1/\sqrt{2} = 0.71$ approximately.

above 8μ but a slight and continuous rise in the curve. Taylor (1942) ascribes the differences between his results and those of Gasser & Grundfest (1939) to differential shrinkage of axon and myelin in their preparations, brought by fixation, paraffin embedding, and staining. However, the fact that the present results, which resemble Taylor's, were obtained from fixed and stained fibres, and the
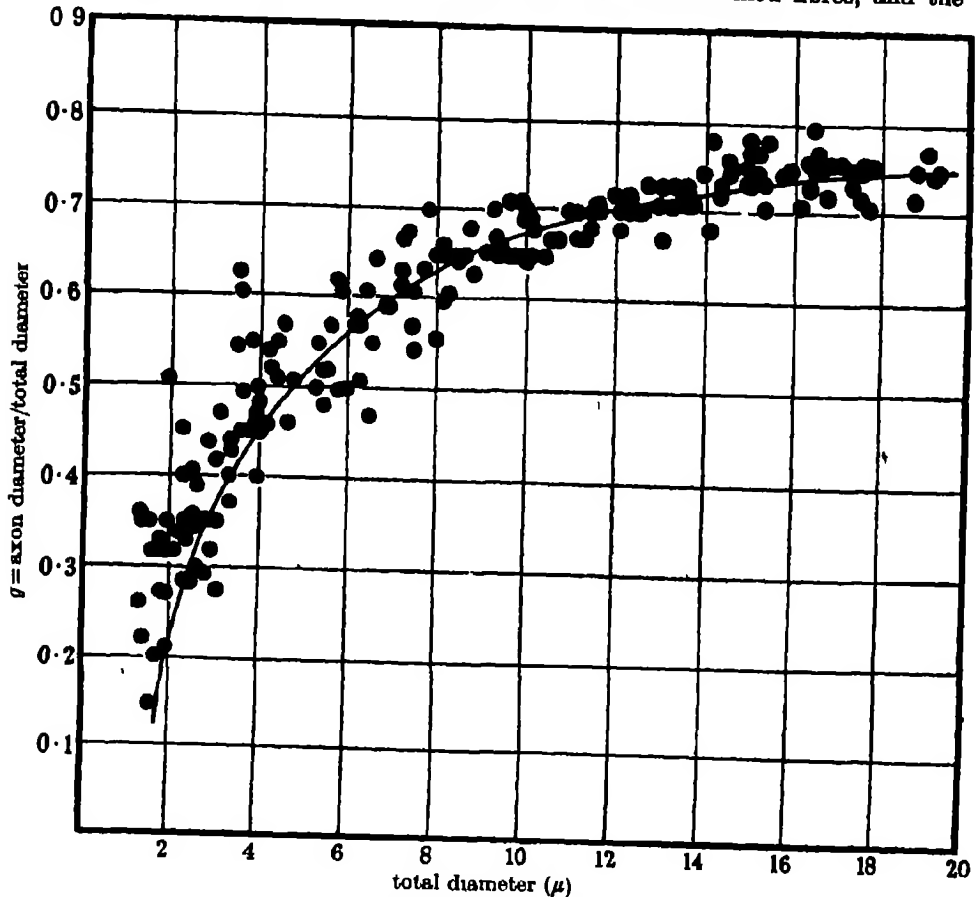

FIGURE 3. Graph of the ratio g against total diameter for 200 selected normal myelinated fibres. The curve was drawn by eye

results of comparative measurements of fixed and fresh nerve fibres given on pp. 328 to 329, indicate that this explanation cannot be the true one. The curve given by Gasser & Grundfest has only a relatively small number of points, possibly too few to give an adequate idea of the shape of the tail of the curve.

All these results, however, show that g is not constant for all fibres, but has a relation to fibre diameter. In other words, consideration of the variation of the ratio g with fibre diameter leads us to the same conclusion as emerged from a consideration of the relative cross-sectional areas of axon and myelin, namely, that the axon is relatively smaller, and consequently the myelin relatively thicker, on the smaller fibres. Both these quantities, then, indicate that there exists

a relationship between the size of a fibre and the dimensions of the myelin sheath which encloses it. However, when we come to determine the exact nature of the relationship between myelin sheath and axon dimensions, neither g nor the *area ratio* provides a sufficiently sensitive index. This fact is perhaps best illustrated by the following example:

(a)

(b)

(c)
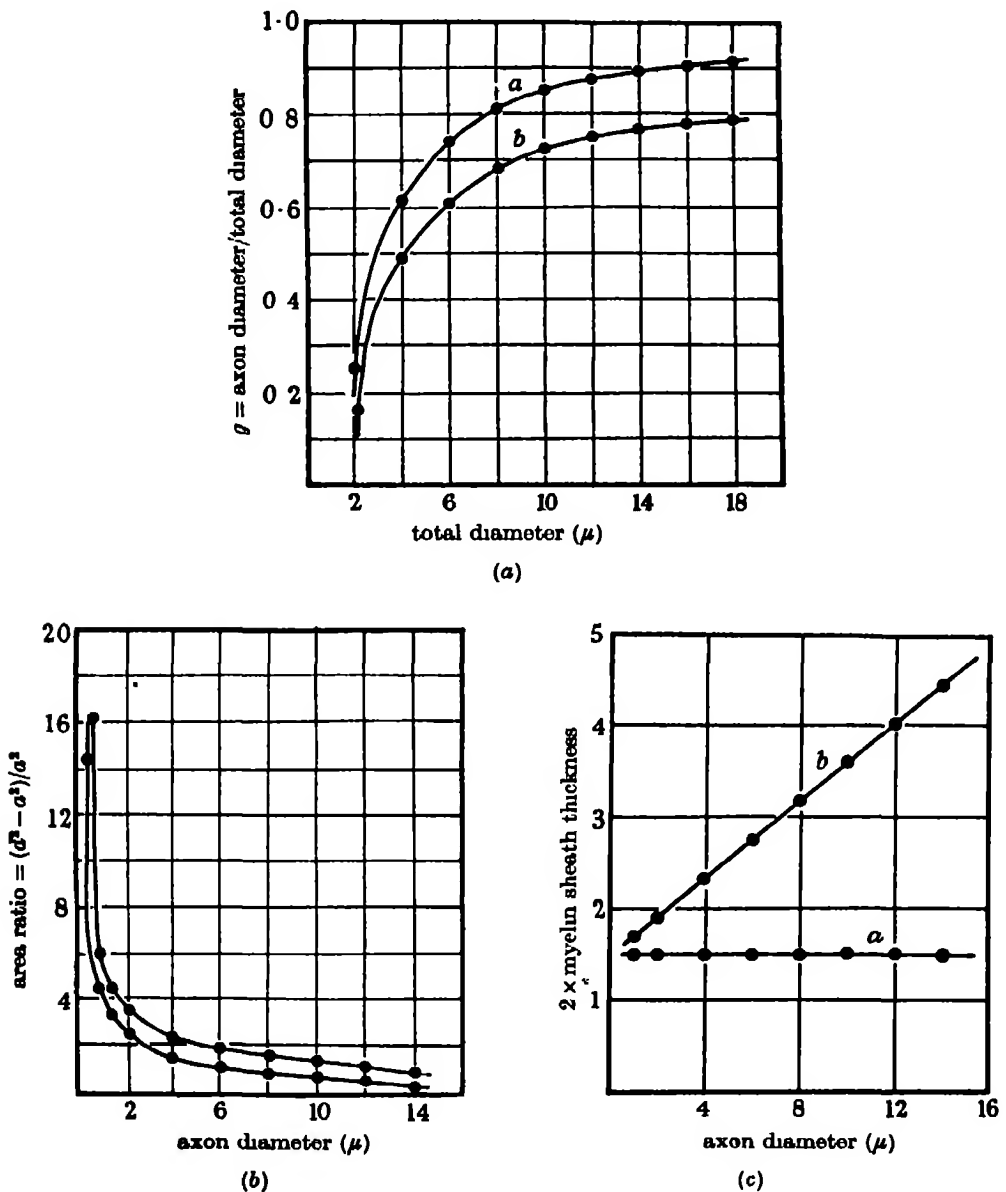
FIGURE 4 (a) to (c). Graphs of (a) g against total diameter, (b) the *area ratio* against axon diameter, and, (c) 2 × sheath thickness against axon diameter, for two arbitrary theoretical relationships of axon diameter and sheath thickness (see text). Line *a*, where the sheath is 0.75μ thick on all fibres. Line *b*, where the sheath thickness = 0.1 × the axon diameter + 0.8.

Figure 4 (a) and (b) give the curves of g against total fibre diameter, and of *area ratio* against axon diameter, for two arbitrarily chosen relations between axon and myelin dimensions. The theoretical cases chosen were: (a) where fibres of all diameters have myelin sheaths of the same thickness, and (b) where sheath thickness varies as an arbitrary linear function of axon diameter. In the case of g (figure 4 (a)) the two curves differ in position, but are both convex upwards and have a gradually rising tail. Thus features of the curve of g noted as significant by Taylor (1942) are implicit in the form of the ratio, and largely independent of the actual relationship between axon and myelin dimensions. Curves of the *area ratio* (figure 4 (b)) in these two cases are even more alike. In fact, given a set of experimental points such as those in figure 2 it would be difficult to be sure which of the above relations gave a curve with the best fit. Thus, even if myelin sheath thickness is to a great degree independent of fibre diameter, a curve of g against total diameter, or of the *area ratio* against axon diameter, will have the same general shape as those obtained.

However, the curve obtained by simply plotting the myelin thickness against the axon diameter, without having recourse to the calculation of ratios, does not suffer from these disadvantages. Figure 4 (c) shows the result of plotting axon diameter (a, see figure 1) directly against $2 \times$ the myelin sheath thickness $(d - a$, see figure 1) in the case of the two theoretical relationships defined above. Were the myelin thickness constant on all fibres, this method of plotting would give a straight line parallel to the base-line (line a in figure 4 (a)) If the sheath thickness varied linearly as the axon diameter, the result would be a straight line inclined at an angle to the base line (figure 4 (c), line b) If the relation between myelin thickness and fibre diameter were non-linear, the result would be a curve, and so on. It will be seen that this method of plotting makes it easier to determine the exact nature of the relationship between myelin thickness and fibre diameter than does either of the others.

Figure 5 is a direct plot of $2 \times$ myelin thickness against axon diameter in the case of the 200 normal fibres measured. Comparing figure 5 with figure 4 (c) it is obvious that all fibres do not have sheaths of the same absolute thickness, neither does sheath thickness vary linearly with axon diameter Nevertheless, sheath thickness shows a strong correlation with axon diameter, and from inspection of the graph it appears that the relationship between the two quantities is best represented by a curve. This curve is perhaps steep at first, though flattening off for fibres with an axon diameter exceeding 1 to 2μ.

In figure 6 the points of figure 5 are shown plotted on a double logarithmic scale. The effect of changing the scale is to straighten out much of the slight curvature apparent in figure 5, and to make it possible that a straight line may fit the data. The dotted line in figure 6 is the regression line drawn to the whole data. Applying the test of goodness of fit mentioned on p. 326 to this line, it was found that the variance ratio had a value of 1·74 which corresponds to a probability of about 0·05. The details of this analysis of variance are set out in table 2. Taking the conventional limit of $P = 0.05$, this means that a straight line is a satisfactory fit to these data. However, this result lies barely within the limits of significance, and it was
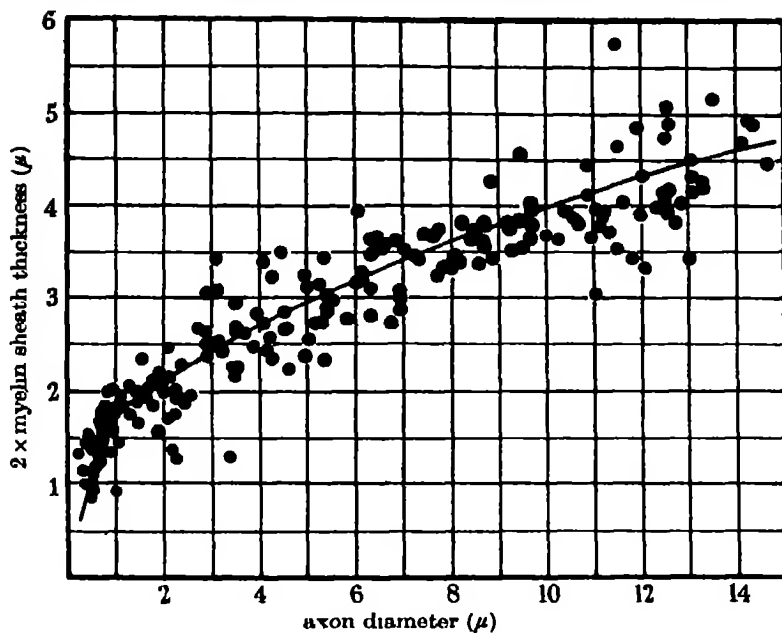
FIGURE 5 Graph of 2 × myelin sheath thickness against axon diameter for 200 selected normal nerve fibres. Curve calculated as explained in text.
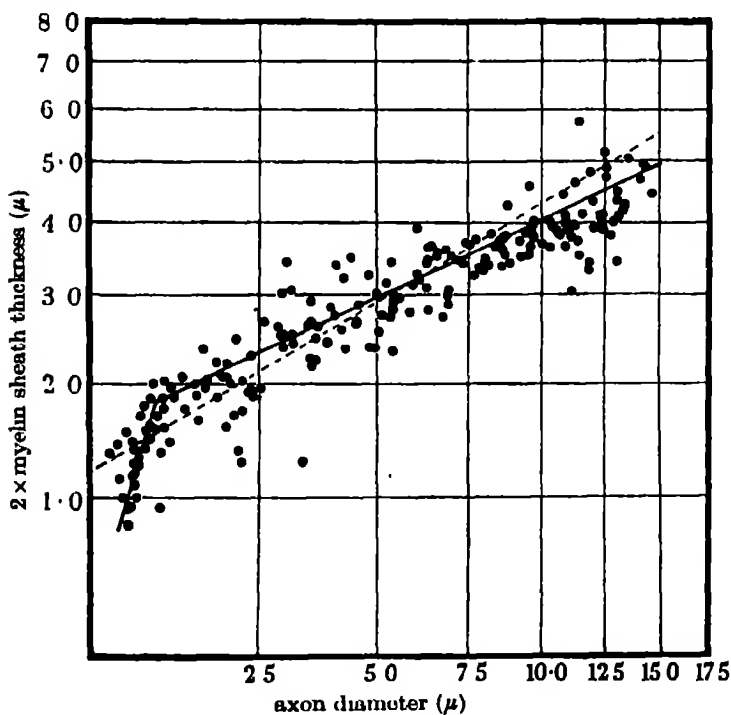

FIGURE 6. Plot of 2 × sheath thickness against axon diameter for 200 normal nerve fibres on a double logarithmic scale. Dotted line = calculated regression line for whole data Continuous lines = regression lines calculated for axons below and above 1μ respectively.

apparent from the calculation that by far the greatest part of the deviation from linearity was due to the points representing fibres with an axon diameter of less than 1μ. Thus a second regression line was calculated after omitting these fibres, and is shown by the second of the two continuous lines in figure 6. Applying the same test of goodness of fit to this second line, it was found that the variance ratio = 1·32, whence $P = > 0·20$, which is well within the limits of significance (for analysis of variance see table 2).

TABLE 2. ANALYSIS OF VARIANCE OF DATA FROM NORMAL NERVE

item	sums of squares	degrees of freedom	mean square	variance ratio	probability
(a) whole data					
variance between arrays due to regression	493 805	1	—	—	—
variance due to deviations from linear regression	13 114	13	1 009	1·74	approx. 0 05
variance within arrays	95 757	165	0 580	—	—
total	602 676	179	—	—	—
(b) omitting points below 1μ					
variance between arrays due to regression	276·474	1	—	—	—
variance due to deviations from linear regression	8 125	11	0·739	1 32	approx. 0·2
variance within arrays	82 501	147	0 561	—	—
total	367 100	159	—	—	—

It was also found possible to fit a straight line to the points excluded above, and this is shown by the first of the two continuous lines in figure 6. When the variances from the two straight lines so obtained were pooled, it was found that they gave an adequate fit to the whole of the data. However, it will be noted that there is a large concentration of points below the line in figure 6, between axon diameters of 7·5 to 12·5μ. These points also lie below the curve in figure 5, which was obtained from the straight lines in figure 6 in the way already described (p. 326). Hence it is probable that the curve giving the best fit for this region actually lies somewhat below that drawn.

From the curve of figure 5 it can therefore be concluded that the absolute thickness of the myelin sheath of nerve fibres varies with the axon diameter. The smallest myelinated fibres have sheaths whose thickness is about 0·5μ. With increasing axon diameter the sheath thickness also increases, at first rapidly until it reaches a value of about 1·0μ at an axon diameter of 1·0μ. At this point there is a kink in the curve, and thereafter the sheath thickness increases less rapidly for each increment of axon diameter. Fibres with axons between 12 and 14μ in diameter thus have sheaths between 2·0 and 2·5μ in thickness.

It is of interest to compare the present curve of $2 \times$ sheath thickness against axon diameter (figure 5) with the curve of sheath birefringence against total diameter given by Taylor (1942; figures 1 and 2). When the sheath birefringence of nerve fibres is measured, the smallest fibres show negative birefringence due to the prevalence

of the form birefringence of the contained protein, while the large fibres, which contain more lipide, show positive micellar birefringence. The transition from the 'proteotropic' to the 'lipotropic' type takes place at a total fibre diameter of about 2μ, and it has been suggested (Schmitt & Bear 1939) that, although there is no discontinuity between them, the proteotropic and lipotropic fibre types correspond to the 'non-myelinated' and 'myelinated' fibres revealed by ordinary histological techniques. Thus Taylor's curve of sheath birefringence against total fibre diameter has the following characteristics: at fibre diameters below 2μ, sheath
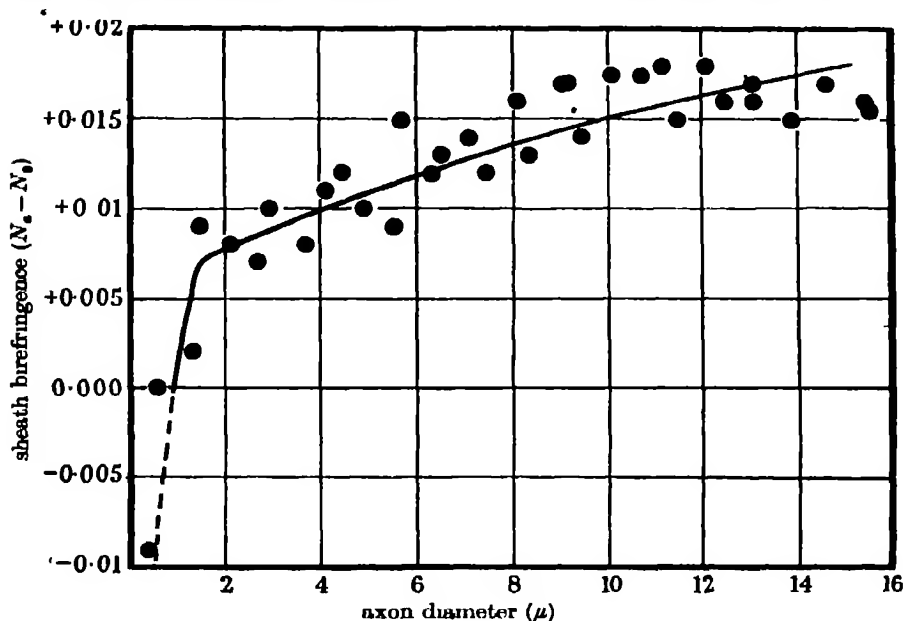
FIGURE 7 Graph of mean sheath birefringence for groups of fibres differing by steps of 0 5μ, plotted against mean axon diameter for the same groups. This graph was obtained by recalculating the data of Taylor (1942), and the curve was fitted by the method described in the text Dotted part of curve = 'proteotropic' fibres.

birefringence is negative, though increasing rapidly with growing fibre diameter, and becoming zero at a diameter of 2μ Above 2μ the sheath birefringence continues to increase rapidly until a total diameter of about 3μ is reached. At this point there is a sharp change in the shape of the curve relating sheath birefringence to fibre diameter, and thereafter sheath birefringence only increases relatively slowly with additional increments of fibre diameter.

In figure 7 the data for mean sheath birefringence given by Taylor (1942, figure 2) for cat saphenous nerve fibres are shown plotted against axon diameter. The values of axon diameter corresponding to the sheath birefringence were obtained by calculation from the curve of g also given by Taylor (1942, figure 4) In figure 7 the dotted part of the curve corresponds to proteotropic, i.e. non-myelinated, fibres. Considering that this curve was obtained from fixed, and Taylor's from fresh, nerve fibres, the resemblance between the shape of the curve in figure 5 and the part of the

curve in figure 7 which refers to lipotropic fibres is especially striking. Both sheath birefringence and sheath thickness at first increase rapidly with axon diameter, roughly in the same proportion. Then, at an axon diameter of 1 to 2μ, there is a sharp change of curvature, so that thereafter both birefringence and sheath thickness increase relatively slowly with axon diameter. The fact that both sheath thickness and birefringence change their relation to axon diameter at about the same place suggests that there is a fundamental change in fibre type at this point. This conclusion can, however, at present be only tentative, in view of the relatively small number of fibres measured in this group. Further experiments are in progress to test this suggestion.

THE RELATION BETWEEN AXON AND MYELIN DIMENSIONS IN REGENERATING NERVES

The axon and total diameters of myelinated nerve fibres were measured 60, 100, 200 and 300 days after operation in the central and peripheral stumps of nerves which had been interrupted by sharp localized crushing with fine smooth-tipped forceps. This operation interrupts the nerve fibres, but leaves the endoneurium largely intact, with the result that the regenerating fibres undergo little criss-crossing in the scar, and travel mostly down the pathways which they occupied before the injury (Young 1942). For this reason a more complete reconstitution of the nerve occurs after crushing than after other types of injury and nerve repair (Gutmann & Sanders 1943) The situation is therefore an ideal one for studying myelin regeneration in the absence of complicating factors such as excessive shunting of fibres into unusual pathways.

Central stumps

60 *days*. Figure 8 is linear plot of axon diameter against $2 \times$ myelin sheath thickness for fibres 15 mm. above the site of crushing, in the case of 165 fibres taken from a central stump 60 days after injury. Fibres with axons ranging in diameter from 0·5 to $12\cdot5\mu$ were present. The largest axons found in this nerve were thus smaller than the largest found in normal nerves ($14\cdot7\mu$, see figure 5). The myelin sheaths of these fibres varied in thickness from $0\cdot60\mu$ on the smallest to about $3\cdot5\mu$ on the largest. The curve relating sheath thickness to axon diameter given in figure 8 is considerably steeper than that of the normal nerve (cf. figure 5), which indicates that the thickness of the sheath of all except the smallest axons is relatively greater than of those of a similar axon diameter in the normal nerve. Gutmann & Sanders (1943) found that the spectrum of total fibre diameters differed little from that of normal nerve at this stage, the number of large fibres, and the mean fibre diameter, being only a little less than normal. In fact, adding together the axon diameter and double myelin thickness of the largest fibre in figure 8 gives it a total diameter of $19\cdot4\mu$, which is about the diameter of the largest fibres in normal nerves. The production of the type of fibre seen in the central stump at 60 days—i.e. fibres with thick myelin sheaths and small axons—from normal fibres of similar total diameter must therefore involve a thickening of the myelin sheaths at the expense of the axons, with little change in total diameter.

100 *days*. At 100 days after crushing, the thickness of the myelin sheaths on all except the largest axons in the central stump was roughly the same as at 60 days. Figure 9 shows the result of plotting 2 × myelin thickness against axon diameter on a linear scale in the case of 158 selected fibres. It will be seen that this curve closely resembles the one obtained 60 days after crushing (see figure 8) up to an axon diameter of 5 to 6μ. Most of the axons larger than this diameter have slightly
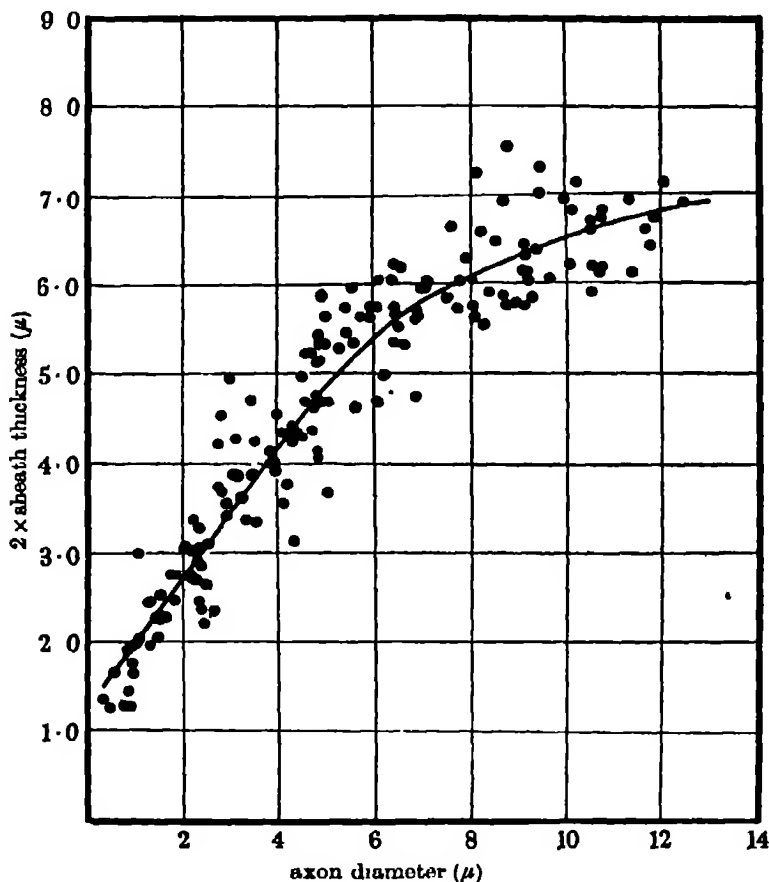
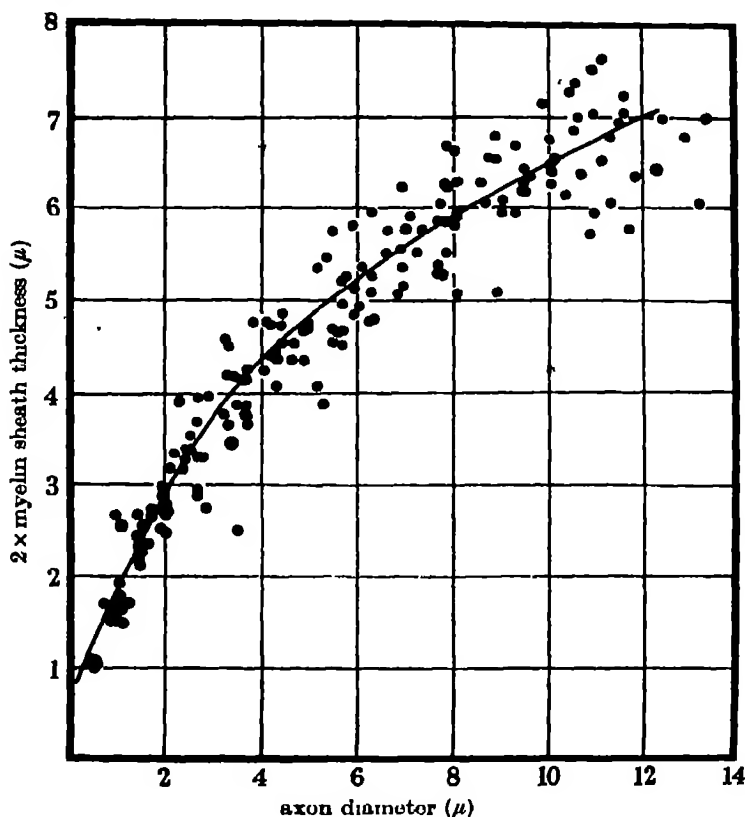FIGURE 8 Graph, on a linear scale, of 2 × sheath thickness against axon diameter for 165 selected myelinated fibres from a central stump at 60 days after crushing. Calculated curve.

thinner sheaths than corresponding ones at 60 days after crushing; however since the points on the graph at 100 days show a much greater scatter than at 60 days it is doubtful whether this difference can be regarded as significant. Much more important is the fact that with a single exception the largest axons found at 100 days had a diameter of less than 11μ, which, with a double sheath thickness of about 5 to 6μ gave them a total diameter of 16 to 17μ. Thus the fibres of the central stump at 100 days have smaller axons than at 60 days, although the thickness of their myelin sheaths may be unchanged, consequently they show a shrinkage in

diameter of the whole fibre compared with the 60-day stage, a fact which was commented upon by Gutmann & Sanders (1943).

200 days. Figure 10 is a linear plot of 2 × myelin sheath thickness against axon diameter in the case of 175 fibres in a central stump taken 200 days after crushing the nerve. It will be seen that the myelin sheaths of the largest fibres are even thicker than at 60 or 100 days, having thicknesses of about 3·0 and 3·5 μ. Total
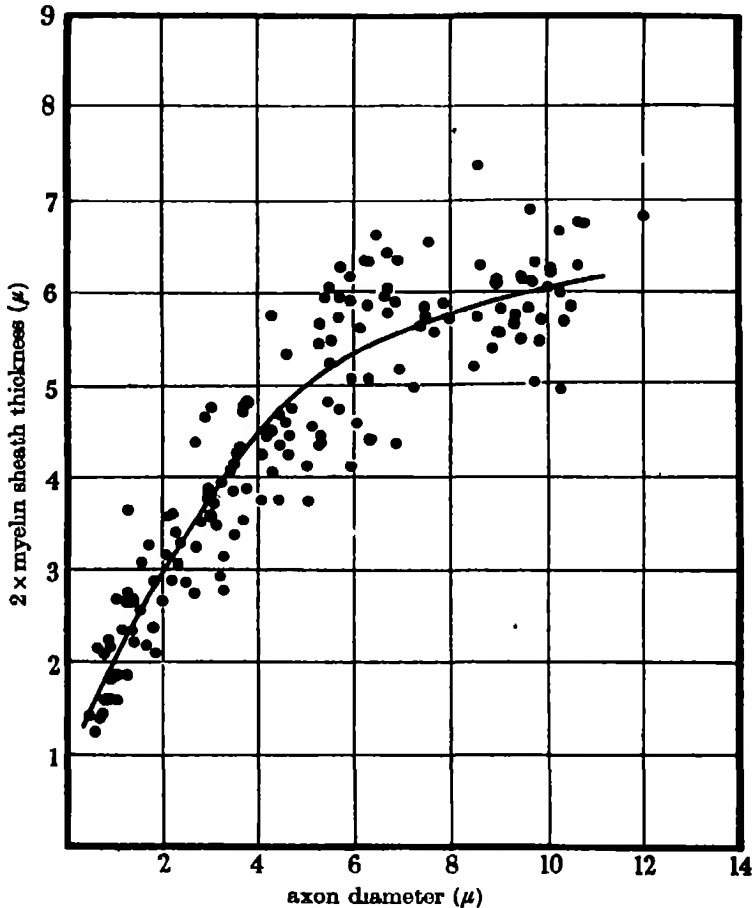
FIGURE 9. Graph, on a linear scale, of 2 × sheath thickness against axon diameter for 158 selected myelinated fibres from a central stump 100 days after crushing. Calculated curve.

diameters, however, at 200 days are greater than at 100 days (Gutmann & Sanders 1943). The curve of figure 10, derived from two lines fitted to a logarithmic plot, shows that between 100 and 200 days after crushing the myelin and axon both increase in size together.

300 days. At 300 days after crushing, the myelin sheaths of all except the smallest fibres in the central stump are thinner than at earlier stages of regeneration, although the sheath thicknesses still remain greater than normal. A linear plot of 2 × myelin sheath thickness against axon diameter for 182 such fibres at 300 days is shown in

figure 11. The largest fibres have sheaths between 2·5 and 3·0μ in thickness. However, their axons are much larger than at earlier stages, so that the total diameter of the fibres is equivalent to those found in normal nerve. In fact, Gutmann & Sanders (1943) found that 300 days after crushing the spectrum of total diameters was restored throughout the nerve.

The curve drawn in figure 11 was calculated in the manner already described from three straight lines fitted to a logarithmic plot.
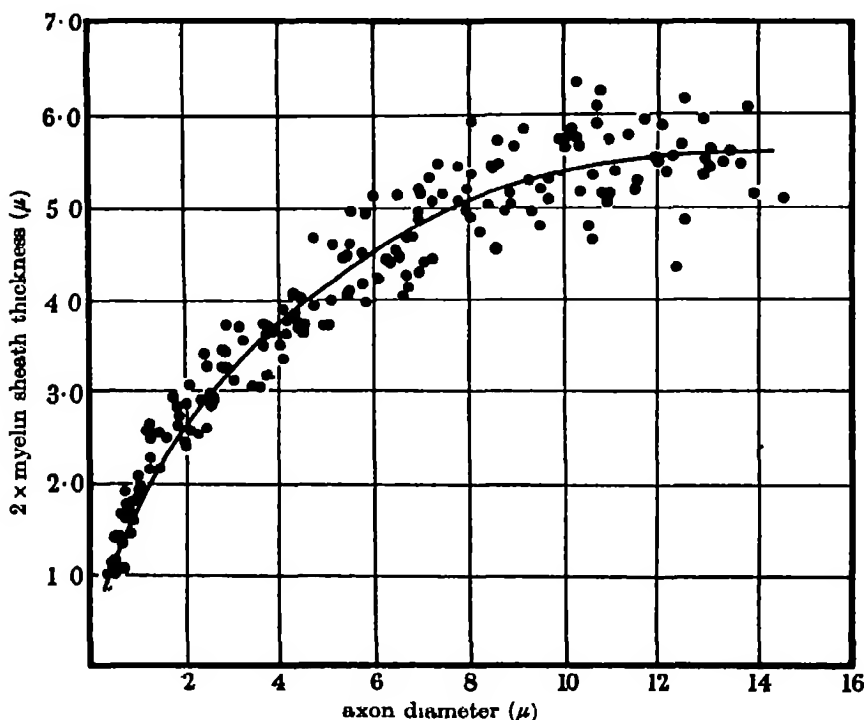

FIGURE 10 Graph, on a linear scale, of 2 × sheath thickness against axon diameter for 175 selected myelinated fibres from a central stump 200 days after crushing. Calculated curve.

Taken together with the results of Gutmann & Sanders (1943) the above measurements indicate that during regeneration there are changes in (a) the total diameter, (b) the axon diameter, and (c) the myelin sheath thickness, of fibres central to the lesion. These changes are summarized in figure 12, where the sheath-thickness-axon diameter curves for normal nerve and central stumps at 60, 100, 200 and 300 days after injury are shown plotted on the same graph The cycle of change in axon, whole fibre, and myelin sheath dimensions during regeneration is best described by first of all considering the changes as they apply to a single large fibre, e.g. one which in normal nerve has a total diameter of, say, 18·5μ, with an axon 14·0μ in diameter, and a myelin sheath whose double thickness is about 4·5μ. During the

first 100 days of regeneration the axon of such a fibre will decrease in size until its diameter is about 10μ. After this the axon will start to increase in size again, although as late as 300 days after injury its diameter will still be only 13μ, less than its original diameter. Parallel with these changes in axon diameter alterations of myelin thickness and total diameter will take place For the first 60 days after injury the shrinkage of the axons is accompanied by a thickening of the myelin, so that at 60 days the double sheath thickness of the fibre described would be about

FIGURE 11. Graph, on a linear scale, of 2 × sheath thickness against axon diameter for 182 selected fibres from a central stump 300 days after crushing. Calculated curve.

6.5μ. At this stage the total diameter is only a little less than normal. Between 60 and 100 days after injury little change in myelin thickness occurs Since shrinkage of the axon is still taking place, this will cause a decrease in total diameter which, in the case of the fibre described will be reduced to about 16μ at 100 days. Between 100 and 200 days there may be a slight increase in sheath thickness, which, coupled with the increase in axon diameter already mentioned, will give the fibre a total diameter of about 18.0μ. Between 200 and 300 days a small increase in total diameter takes place, accompanied by a considerable increase in axon diameter, and a decrease in sheath thickness. At 300 days after injury, therefore, the fibre described has a total diameter of 18.5μ—the same as its original total diameter; an axon diameter of 13.0μ, and a sheath thickness of 5.5μ. Three hundred days after injury therefore, while the total diameter of the fibre has been restored, the axon is still smaller, and the myelin sheath thicker, than in the normal nerve. It is possible

that at still later stages a restoration of the normal relation between sheath thickness and axon diameter will occur.

While the kind of changes described above for a single large fibre are typical for the whole range of fibre sizes, fibres of different sizes differ in the relative extent to which they are affected. This fact is shown by the curves in figure 12. Were all fibres affected to the same extent, the result of increasing myelin thickness at the expense of axon diameter would be merely to displace the normal curve, not to change its shape. Figure 12 shows, however, that the curves drawn for these four stumps differ markedly in shape from the normal curve, except in the case of axons with
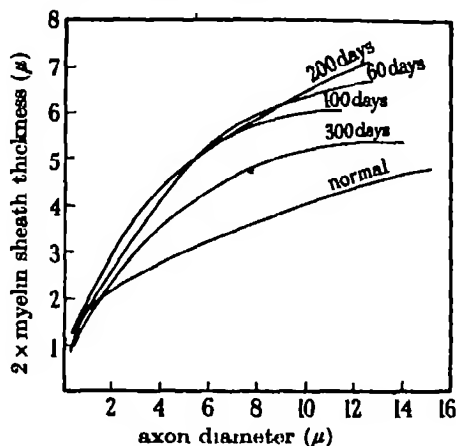
FIGURE 12. Summary of data from central stumps. Calculated curves of 2 × sheath thickness against axon diameter in the case of (1) normal nerve; (2) central stumps 60, 100, 200 and 300 days after crushing.

a diameter of less than 1μ. However, so few of these small fibres were measured in the central stumps, that it is impossible to say anything definite about the behaviour of fibres in the range of sizes covered by this part of the curve Above an axon diameter of 1μ, however, all four central stump, curves are at first steeper than the corresponding part of the normal curve. At still greater axon diameters (above 8μ) the central stump curves flatten out, so that in this region they are either slightly steeper (200 days), parallel to (60 days), or show a more gradual slope (300 days*) than the normal curve. This means that the changes in axon and myelin dimensions must be relatively greater for small than for larger fibres. The theoretical implications of this finding are further discussed below (see p. 353).

Peripheral stumps

The axon diameters and sheath thicknesses of selected fibres were measured 10 mm. below the lesion in the peripheral stumps of the same four nerves whose central stumps are described above, 60, 100, 200 and 300 days after crushing the nerve.

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* The tail of the curve at 100 days shows a more gradual slope than the normal curve in this region. However, as already stated (p. 341), the scatter of the individual points in this graph is so great as to render doubtful any conclusion drawn from this part of the curve.

60 *days.* Figure 13 shows a linear plot of 2 × sheath thickness against axon diameter in the case of 100 fibres taken from a peripheral stump 60 days after crushing. The largest fibres found had total diameters of about 9·5 μ, these had axons about 6 μ in diameter, with myelin sheaths about 1·5 μ thick, similar to those enclosing axons of the same diameter in normal nerve. When plotted on double logarithmic coordinates the curve of sheath thickness against axon diameter could be clearly resolved into two straight lines, intersecting at an axon diameter of about 2·5 μ and the curve derived from these lines shows that, up to a diameter of about 2·5 μ, the increase of sheath thickness with axon diameter is faster than in normal
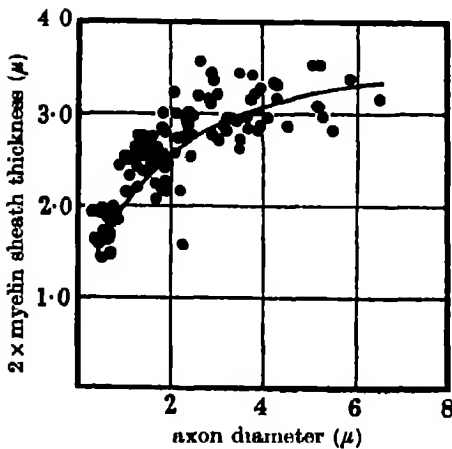
FIGURE 13. Graph, on a linear scale, of 2 × sheath thickness against axon diameter for 100 selected fibres from a peripheral stump 60 days after crushing. Calculated curve
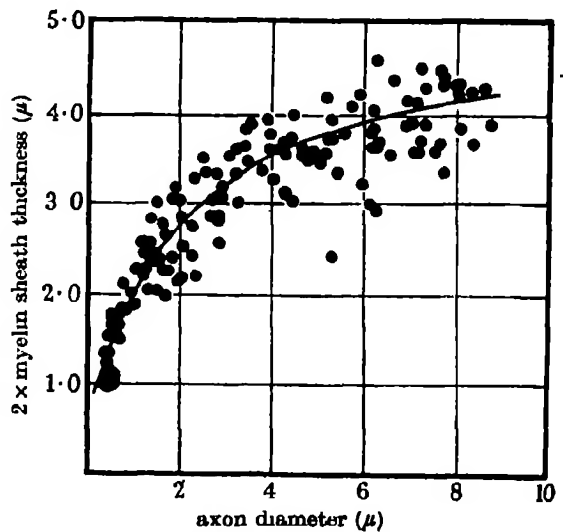
FIGURE 14. Graph, on a linear scale, of 2 × sheath thickness against axon diameter for 133 fibres from a peripheral stump 100 days after crushing. Calculated curve.

nerve. Above this diameter the slope is more gradual than that of the normal curve. It follows that the largest and smallest axons in the peripheral stump at 60 days have sheaths of the same thickness as those enclosing axons of the same diameter in normal nerve. Intermediate-sized axons, particularly those with a diameter of about 2·5 μ, seem to have somewhat thicker sheaths than the corresponding axons in normal nerve.

100 *days.* The relationship of 2 × myelin sheath thickness and axon diameter in a peripheral stump at 100 days is shown in figure 14. The total number of fibres measured was 133. The axons have a maximum diameter of 8 μ, the largest fibres bearing a myelin sheath 2 to 3 μ in thickness. The smallest fibres have axons which are less than 0·5 μ in diameter, with sheaths about 0·5 μ thick. On all but the very smallest fibres the myelin is thicker than on normal nerve fibres of corresponding axon diameter. It will be seen that, with increasing axon diameters, the sheath thickness at first increases relatively rapidly up to a value of 1 μ on axons with

a diameter of 1μ. Thereafter, until the largest axons (9μ) are reached, smaller and smaller additions of myelin accompany successive increments of axon diameter. The sheath-thickness-axon diameter curve at 100 days thus resembles the 60-day curve in its general form. Fibres with axons greater than 1 to 2μ in diameter, however, have thicker sheaths at 100 than at 60 days. Moreover, there are larger axons present at 100 days than at 60 days.
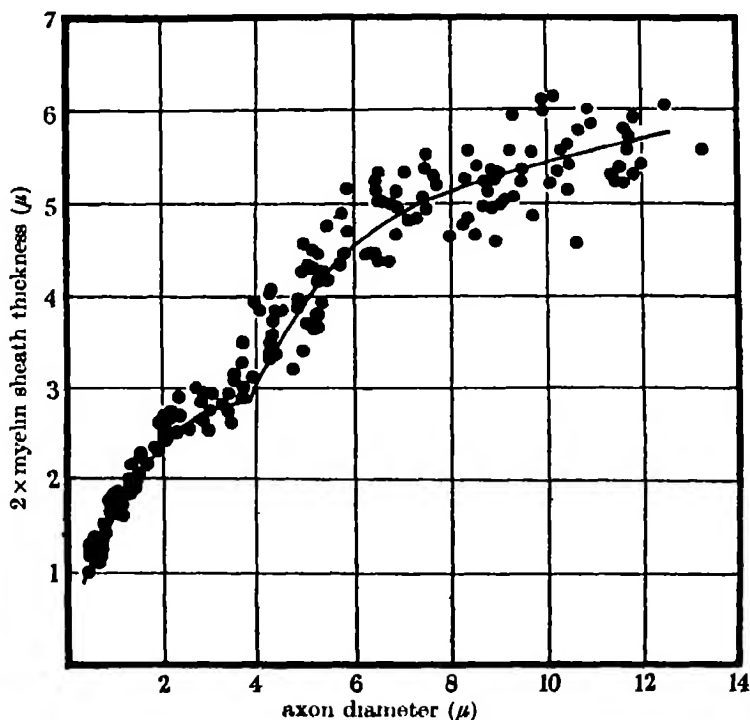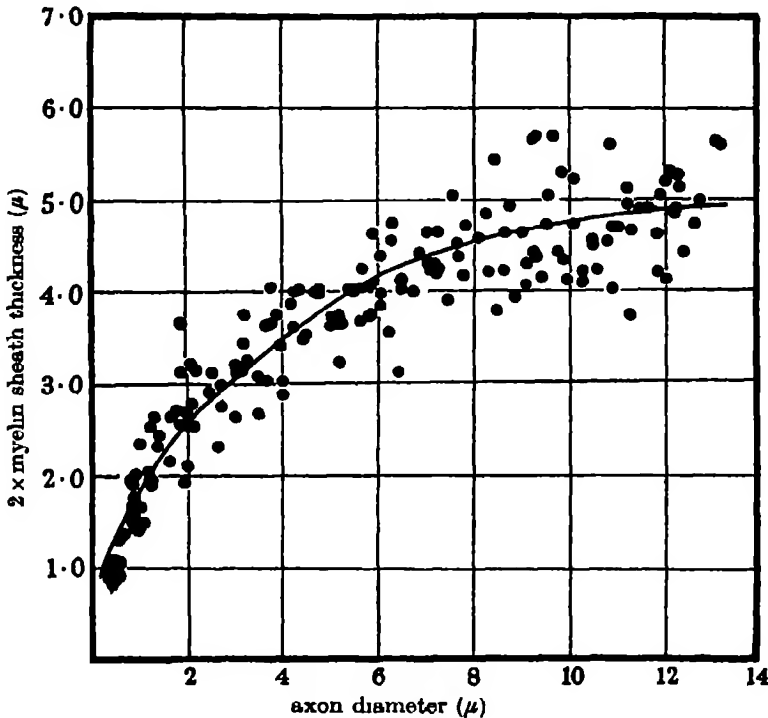

FIGURE 15. Graph, on a linear scale, of 2 × sheath thickness against axon diameter for 168 fibres from a peripheral stump 200 days after crushing. Calculated curve.

200 days. Two hundred days after crushing, the fibres in the peripheral stump still have myelin sheaths which are thicker than on the corresponding axons in normal nerve. Figure 15 is a linear plot of 2 × myelin sheath thickness against axon diameter for 168 selected fibres in a 200-day peripheral stump. The largest axons have a diameter of 12 to 13μ and bear myelin sheaths 2·5 to 3·0μ in thickness. The smallest axons, which are less than 0·5μ in diameter, have sheaths about 0·5μ thick. Between these two extremes, the curve relating myelin sheath thickness to axon diameter has a serpentine form. At first, sheath thickness increases rapidly with increasing axon diameter up to an axon diameter of about 2μ. At this point the curve flattens off, so that, at an axon diameter of 3·5μ, 2 × sheath thickness has a value of about 2·5μ. At this point the axons have sheaths of the same thickness as the corresponding axons in normal nerve. Indeed, over its whole range below this axon diameter the curve is not much different from the normal curve, in view of the small number of fibres measured. Above an axon diameter of 3·5μ, however,

the curve resumes its initial steep slope, flattening off again at an axon diameter of about 6μ into a gradually rising tail which is roughly parallel to the tail of the normal curve. It was, at first, doubtful whether the sharp change in slope of the curve which occurs at about 3·5μ was in fact a real change, or due to the data selected. When the curve is plotted on double logarithmic coordinates, however, the kink at 3·5μ is still very apparent and it was found that no less than four straight lines were required to give an adequate fit by the criteria adopted

FIGURE 16. Graph, on a linear scale, of 2 × sheath thickness against axon diameter for 173 fibres from a peripheral stump 300 days after crushing. Calculated curve

300 *days*. Except in the case of the smallest fibres, the myelin sheaths of axons in the peripheral stump 300 days after crushing are still thicker than those of axons of corresponding diameter in normal nerve. A plot of 2 × myelin sheath thickness against axon diameter on a linear scale (figure 16) shows that the largest axons have diameters slightly greater than the corresponding ones at 200 days. The myelin sheaths enclosing these largest fibres, however, are thinner than on corresponding fibres in the 200-day stump (2·0 to 2·5μ, as compared with 2·5 to 3·0μ). While the sheaths of the largest fibres in the peripheral stump 300 days after crushing are only slightly thicker than normal, the sheaths of moderate-sized axons (diameters 6 to 8μ) are relatively much thicker than their normal counterpart (about 2·0 as compared with 1·5μ). Indeed the sheath-thickness-axon curve of the peripheral stump 300 days after crushing has quite a different shape from the normal curve. For the smallest fibres the two curves have a similar slope. Then,

when the normal curve undergoes its sharp change of curvature at an axon diameter of 1μ, the 300-day curve remains steep and diverges from it. The 300-day curve later flattens out at an axon diameter of about 6μ, and eventually comes to have a more gradual slope than the normal curve. The result of this is that in the region of the largest fibres, the two curves tend to approach one another.
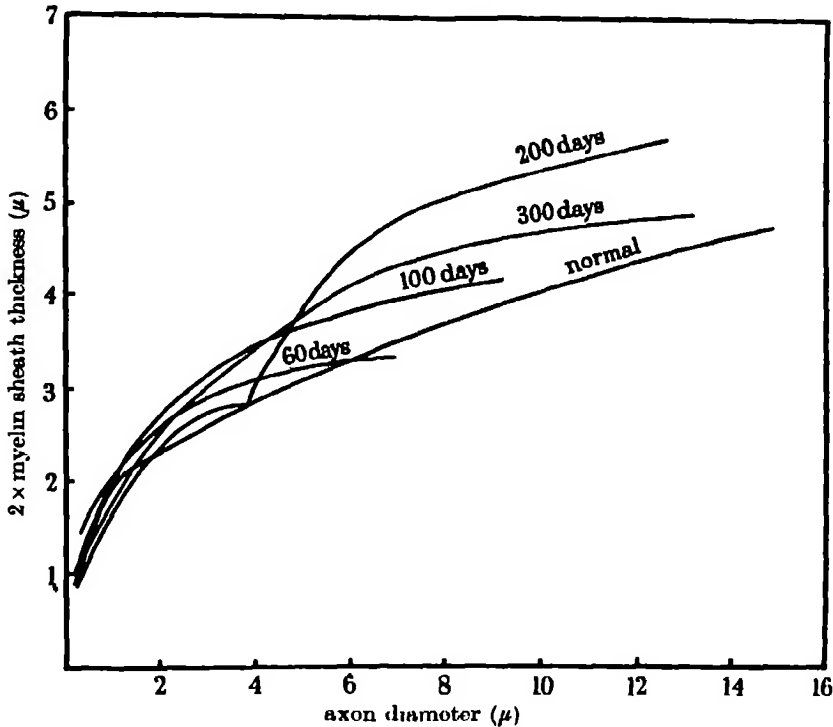
FIGURE 17. Summary of data from peripheral stumps. Calculated curves of $2 \times$ sheath thickness against axon diameter in the case of (1) normal nerve, (2) peripheral stumps 60, 100, 200 and 300 days after crushing

These data from peripheral stumps, therefore, show that new-developed myelin sheaths 60 days after injury are not much thicker than in normal nerve on fibres of corresponding diameter. At later stages the myelin becomes thicker than normal on all fibres, reaching its maximum thickness at about 200 days after injury. At 300 days a decline in the thickness of the sheath on the largest fibres is apparent, and it is possible that the normal relation is eventually restored. Experiments on still later stages of regeneration are in progress to test this conclusion.

In figure 17 the curves for the above four peripheral stumps are shown plotted on the same graph as the curve from normal nerve. The four curves form a series, first of all diverging from the normal curve, and then, at the latest stage, approaching it again. The 60-day curve diverges least from normal; the 100-day is more divergent, and the 200-day more so still, while the 300-day curve is more like normal again. In this, and in other respects, the curves resemble those taken from the central stumps at these times (see figure 12). For example, both central and peripheral stumps at

200 days have myelin sheaths which are thicker than normal. Moreover, the shape of their curves differs from the normal curve in much the same way. Curves from both stumps are steeper than the normal curve at first, and later flatten out. In every case, however, the curve from the peripheral stump is intermediate in position between that of its own central stump and the normal curve.

These observations suggest the possibility that the thickness of the myelin sheath developed by a regenerating nerve fibre is to some extent related to the thickness of the sheath on the same fibre central to the lesion. Speidel (1935 and earlier papers) has emphasized the fact that new myelin only develops on what he terms 'myelin-emergent' fibres, so that if the development of myelin on a regenerating fibre is controlled by whether it is myelinated above the lesion, it is possible that the central stump myelination in some way determines the thickness of the sheaths developed in the periphery. This possibility is strengthened by the fact that remyelination spreads away from the central stump as a wave-front down the nerve (Sanders & Young 1942).

DISCUSSION

The sheath structure of normal nerve fibres

The most remarkable feature of the results obtained from measuring the sheaths of normal nerve fibres during the present work is the striking correlation which exists between sheath thickness and birefringence. When plotted against axon diameter, the curves of myelin thickness for 'myelinated' and sheath birefringence for 'lipotropic' fibres resemble one another very closely (see figures 5 and 7). So similar are they, that, provided the scales chosen are the same, the application of the correction factor for shrinkage, given on p 329, to the data for sheath thickness and axon diameter renders the two curves practically identical in slope and position. Schmitt & Bear (1937, 1939) and subsequent workers interpret their curves of sheath birefringence as showing a continuous change in sheath composition with increasing fibre diameter, more lipide than protein is added for each increment of diameter The micellar birefringence of this extra lipide eventually outweighs the form birefringence of the protein in the sheath, and causes a continuous change of birefringence in the positive direction However, it is not wholly clear whether this change in sheath composition is to be regarded as due to an alteration in the fundamental ultrastructural pattern upon which the sheath is built up, or to a change in the number of units of pattern which the sheath contains. X-ray diffraction studies on whole nerves have shown that there is a fundamental spacing, due to the myelin sheath, of about 185 Å. in mammalian nerve (Schmitt, Bear & Palmer 1941). Comparisons of the diffraction spacings given by wet and dry nerves, and mixtures of nerve lipides, indicate that, of this 185 Å. spacing, 134 Å. is due to lipide, and 25 Å. and 26 Å. probably due to protein and water respectively. We can thus consider the myelin sheath as consisting of concentrically wrapped layers 180 Å thick, each layer containing proportionately more lipide than protein. In addition the sheath may contain relatively large interspersed channels of aqueous

phase, which may explain the readiness with which it sometimes breaks into lamellae (Young 1945). However, since the X-ray analyses were made on whole nerves, it is impossible to say whether the structure described is typical of all fibres in the nerve. Nevertheless, from the similarity between the birefringence and sheath thickness curves we can infer that the structural pattern revealed by X-ray analysis is probably characteristic for all the fibres present. For if we assume that increase in thickness occurs by the addition of identical unit layers of myelin, and not by a fundamental change in ultrastructure, then, for every extra layer, more lipide than protein will be added, and the result will be to produce a change in birefringence paralleling the change in thickness. The present results, therefore, taken in conjunction with those of Taylor (1942), indicate that the myelin sheaths of mammalian nerve fibres of all sizes are similar in the fundamental pattern upon which their ultrastructure is built up, but differ in the number of units which they contain.

Unfortunately this argument no longer holds when applied outside the mammals. We have no evidence that the fundamental unit upon which the sheath is built is the same size in other groups Indeed, there is some evidence that it varies. For example, the X-ray identity period of frog nerve is 171 ± 5 Å, which indicates a possible difference from the mammals (Schmitt, Bear & Palmer 1941). Moreover, in the squid giant fibre the sheath is 3μ thick, and yet shows negative form birefringence (Schmitt, Bear & Young 1937), which is never seen in mammalian sheaths of this thickness. Similarly, earthworm giant fibres have sheaths up to 10μ thick, but are much less positively birefringent than the far thinner sheaths of the largest fibres in mammalian peripheral nerves (Taylor 1940), a fact which indicates that the proportion of lipide to protein must differ greatly in the two groups. Knowledge of the variation of sheath thickness and birefringence with axon diameter, coupled with X-ray diffraction data from as large a number of fibre types as possible, should however provide the information necessary to frame a theory of the effect exercised by sheath structure on the physiological activity of nerve fibres.

Changes in sheath thickness during regeneration

The present work has shown that 15 mm. proximal to a lesion the myelin grows thicker during the first days of regeneration, only growing thinner again after regeneration has been going on for a considerable time. This change in sheath thickness is accompanied by (1) a shrinkage followed by an expansion of the contained axon, and (2) a shrinkage followed by an expansion of the whole fibre. Gutmann & Sanders (1943) studied the shrinkage and expansion of whole fibres in the central stump. They found that, for the first 130 days of regeneration after crushing, the fibres in the central stump undergo a decrease in total diameter At the same time as this is taking place in the central stump, the fibres of the peripheral stump are steadily increasing in diameter. Gutmann & Sanders (1943) give a curve (their figure 3) to illustrate this phenomenon, in which the 'size factors' for central and peripheral stumps of various ages were plotted against time. These size factors (see Gutmann & Sanders 1943, p. 492, for method of calculation) were a measure of

the relative volume occupied by nerve fibres in unit lengths of the central and peripheral stumps.

Gutmann & Sanders (1943) interpret their results as showing that an outflow of axoplasm from the central stump fibres takes place, causing them to decrease in diameter, and that this outflow is responsible for the increase in diameter of the peripheral axons which accompanies the central decrease. Bodian & Mellors (1945) have criticized this interpretation. They point out that the volume of a nerve axon normally greatly exceeds that of the cell which gives rise to it (in a motoneuron supplying the gastrocnemius in *Macacus rhesus* the total volume of the axon is about 250 × that of the cell body) and so the small diminution in diameter central to the lesion observed by Gutmann & Sanders cannot account for the whole mass of the regenerated axon. It is agreed that outflow alone cannot account for the whole mass of axoplasm contained in a completely reconstituted axon, but it is maintained, and re-examination of the data of Gutmann & Sanders (1943) in terms of axon diameters shows, that the diminution in diameter of the central axons provides a sufficient volume of axoplasm to account for the amount of axon which appears peripherally between 0 and 100 days of regeneration. Using the present results combined with the data of Gutmann & Sanders (1943) we have calculated *size factors* in different crushed nerves, using *axon* instead of *total fibre* dimensions in the calculations. This has enabled us to obtain a figure representing the relative volume of axoplasm in unit lengths of the two stumps at different times after injury.* The two curves obtained in this way are shown in figure 18. The figure of 0 days in the central stumps was obtained from the data for normal nerve. The point on the base-line for peripheral stumps represents the day before the arrival of the first axon tips at this level (approximately 7 days), assuming a rate of advance of axon tips of 4·4 mm./day and a latent period in the scar of 5·5 days (see Gutmann, Guttmann, Medawar & Young 1942). In the central stump the amount of axoplasm at first declines rapidly for about 90 days, after which it begins to increase. At 300 days after injury it is approaching, but has not yet reached, the normal value. In the peripheral stump there is a continuous increase in the amount of axoplasm, so that after 200 to 300 days the relative volume per unit length is about equal in the two stumps.

The simplest explanation of these changes in axoplasm content is that for the first 90 days of regeneration the increase of axon volume in the peripheral stump results from a flowing out of axoplasm from the fibres in the central stump. Consider one of the largest fibres in a normal nerve, one with an axon diameter of 15μ and a total diameter of 20μ. In the present experiments the nerves were crushed 80 mm. above the muscle entry, and approximately 100 mm. below the

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* The method used was as follows. The fibres in the normal nerve and the central, and peripheral stumps at 60, 100, 200 and 300 days were divided into 2μ groups and the mean axon diameter for each group calculated. The axon diameters at 70 days were assumed to be the same as at 60 days, and those at 90 days the same as at 100 days. Those at 130 days were taken as the mean of corresponding groups at 100 and 200 days, while those at 250 days were the mean of corresponding groups at 200 and 300 days. From these mean axon diameters the size factors were calculated from the data of Gutmann & Sanders (1943) by the method described in their paper.

cord. At 100 days of regeneration the largest axons found in the central stump had a diameter af about 12μ. Assuming that the diminution in fibre diameter exists all the way up to the cord, the difference in volume between such a regenerating axon and the normal axon from which it is derived would be $\pi \times 10^5 \times (7 \cdot 5^2 - 6^2)$ = approximately $6 \cdot 4 \times 10^6 \mu^3$. Immediately below the lesion the largest fibre has an axon diameter at 100 days of about $8 \cdot 5\mu$. 80 mm. lower down, at the muscle entry, we may estimate that such a fibre has an axon diameter of $6 \cdot 0\mu$. The volume of such a fibre in the peripheral stump can be easily calculated to be
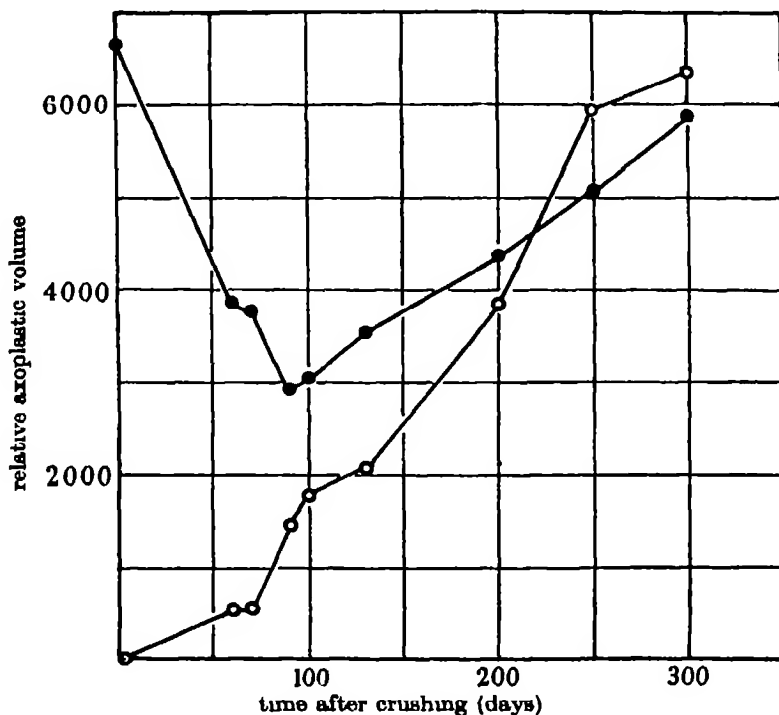
FIGURE 18. Graphs to show the 'relative axoplasmic volume' (see text) in the central and peripheral stumps at different times after crushing the nerve. ● central stump values; ○ peripheral stump values.

approximately $3 \cdot 3 \times 10^6 \mu^3$, a volume actually less than that lost by the fibre in the central stump which gave rise to it. The first 90 days of regeneration, therefore, can be regarded as due to outflow only, the data do not indicate that there is any protein-forming system either at the tip of the advancing axon, or within the expanding peripheral axon in the more proximal regions of the peripheral stumps. Further evidence that outflow of some sort is taking place can be obtained by a study of the behaviour of fibres of different sizes in the central stump. The sheath-thickness-axon diameter curves for central stumps have a different shape from the normal curve, which suggests that the axons of small fibres decrease in diameter proportionately more than those of large fibres (see p 345). In the initial stages the streams of axoplasm from both large and small fibres have to flow the same distance

in order to reach the end organ. The amount of axoplasm required to produce fibres of equal length will deplete small fibres relatively more than large ones.

From 90 days onwards, however, the axons in both central and peripheral stump increase in diameter together. Unless it is found that within this period a protein-forming system appears somewhere within the peripheral stretch of fibre, we can regard this secondary enlargement of axons throughout the nerve as being due to a further flow of axoplasm down the fibre, this new axoplasm being manufactured by the nerve cell. An analysis of the process of chromatolysis by the technique of ultraviolet light microspectroscopy (Gersh & Bodian 1943; Hyden 1943) has shown that this process involves the absorption of water by the cell and the disappearance of ribonucleotide, which is presumably a constituent of the Nissl substance, from the cytoplasm. The whole process is regarded by Hyden (1943) as a mobilization by the cell of its resources before the beginning of protein synthesis, which is thought subsequently to take place at the nuclear membrane. Thus there is some evidence that after nerve interruption the nerve cell is at first inactive and only begins to make protein at a relatively late stage. It is therefore suggested that the provision of new axons in the periphery, which starts very early after a nerve interruption, probably takes place by the following process· the advance of new axons down the peripheral stump and their initial thickening is brought about by the outflow of axoplasm from the stretch of intact fibre above the lesion, possibly aided by a pressure in the fibre resulting from the absorption of water by the cell (Gersh & Bodian 1943; Young 1944); subsequently, the nerve cell starts to manufacture fresh axoplasm which passes down the depleted axons and restores them to their original diameters.

Not all the depletion of the central axons, however, is necessarily due to outflow of axoplasm. Weiss, Edds & Cavanaugh (1945) and Sanders & Young (1945, 1946) have compared the fibres in the central stumps of regenerating nerves which were prevented from making peripheral connexions with those of nerves in which connexion was allowed, and found that the central stump fibres diminished in diameter to a greater extent in the unconnected nerves. The fibres in the peripheral stump, however, had a greater volume in the peripherally connected nerves, so that the greater diminution in diameter of the central stump fibres of the unconnected nerves could not be accounted for on the grounds that greater outflow had taken place from them. It is therefore probable that until connexion with the periphery is achieved there is an actual atrophy of central stump fibres in addition to loss of axoplasm through outflow. Further work, however, is necessary before the proportions of the central diminution in diameter due to atrophy and outflow respectively are established.

The above changes in axon diameter are also accompanied by alterations in the myelin sheath thickness and the total diameter of the fibre. In the normal nerve fibre the myelin is closely bounded on its outer surface by the Schwann cell, the neurilemma and the endoneurium, the collagen fibres of the latter forming the walls of a tube surrounding the nerve fibre. This collagenous tube probably has a certain rigidity compared with the other constituent elements of the nerve fibre, since it exercises a restrictive influence on the swelling fibres in the periphery during re-

generation, and probably controls the final size which they may attain (Gutmann & Sanders 1943; Sanders & Young 1944). In the normal fibre the axoplasm is probably under a slight positive pressure, perhaps a 'turgor' pressure from the cell (Young 1944). This pressure will tend to expand the axon, and since the fibre is contained within a more rigid endoneurial tube, this tendency of the axon to expand must be opposed in some way so that a balanced system is maintained. It has recently been suggested (Young 1945) that the internodal myelin segments can be considered as deformed droplets, the shape of the nodes of Ranvier (the ends of the drops) being consistent with the view that the myelin wets the axon but not the tube wall. We can therefore consider the normal axon as being in equilibrium with its own myelin sheath, a concept which is helpful in interpreting the changes in myelin sheath thickness seen during regeneration. When the axon is severed the internal pressure is released, the axoplasm flows out, and the pressure on the myelin sheath is released, so that the droplets which form the internodal segments tend to round up under surface tension. Such behaviour should be accompanied by retraction of the myelin at the nodes, a phenomenon which has actually been observed to take place (Young 1944), the form of the droplets of retracted myelin being determined by the interfacial tensions between the myelin, the axon, the interstitial fluid, the tube wall and the elasticity of the tube wall. Further outflow would then tend to make the whole tube contract in diameter. Such a process would cause a sequence of changes in the dimensions of axon, myelin, and whole fibre very like those already described.

Similar ideas can be applied to interpret the conditions seen in the peripheral stump. Here the new, expanding, fibres are at first smaller than the tubes in which they are running, the space between the tube wall and the fibres they contain being occupied by the Schwann cell columns, together with macrophages. When the fibres develop myelin sheaths the latter become relatively thicker than those on fibres of similar diameter in normal nerve. As the axons expand, the myelin sheaths they bear are reduced in thickness so that the normal relation may be eventually restored. Presumably, the effect of the continual flow of fresh axoplasm down the fibre is to increase its diameter against the gradually increasing resistance provided by the tube and its content of Schwann cells, until equilibrium between the opposing forces is attained. An effect of this will be to flatten out the myelin droplets, elongating them at the expense of their thickness. The above changes in the central and peripheral stumps are summarized in figure 19.

The actual process of remyelination is thus an extremely complex phenomenon in which many factors are involved. Whether a fibre develops a stainable myelin sheath at all depends presumably upon its diameter (Duncan 1934) and whether it is a 'myelin-emergent' fibre (Speidel 1932). The subsequent adjustment in thickness of the sheath is shown by the present work to depend both upon the diameter of the axon bearing it, and the size of the Schwann tube in which it runs. Thus all factors which influence fibre diameter, such as the presence or absence of peripheral connexion (Sanders & Young 1945) will also affect myelin sheath thickness. There are no doubt further factors which have not been dealt with here. For example, it is not known what changes in myelin ultrastructure accompany growth of the
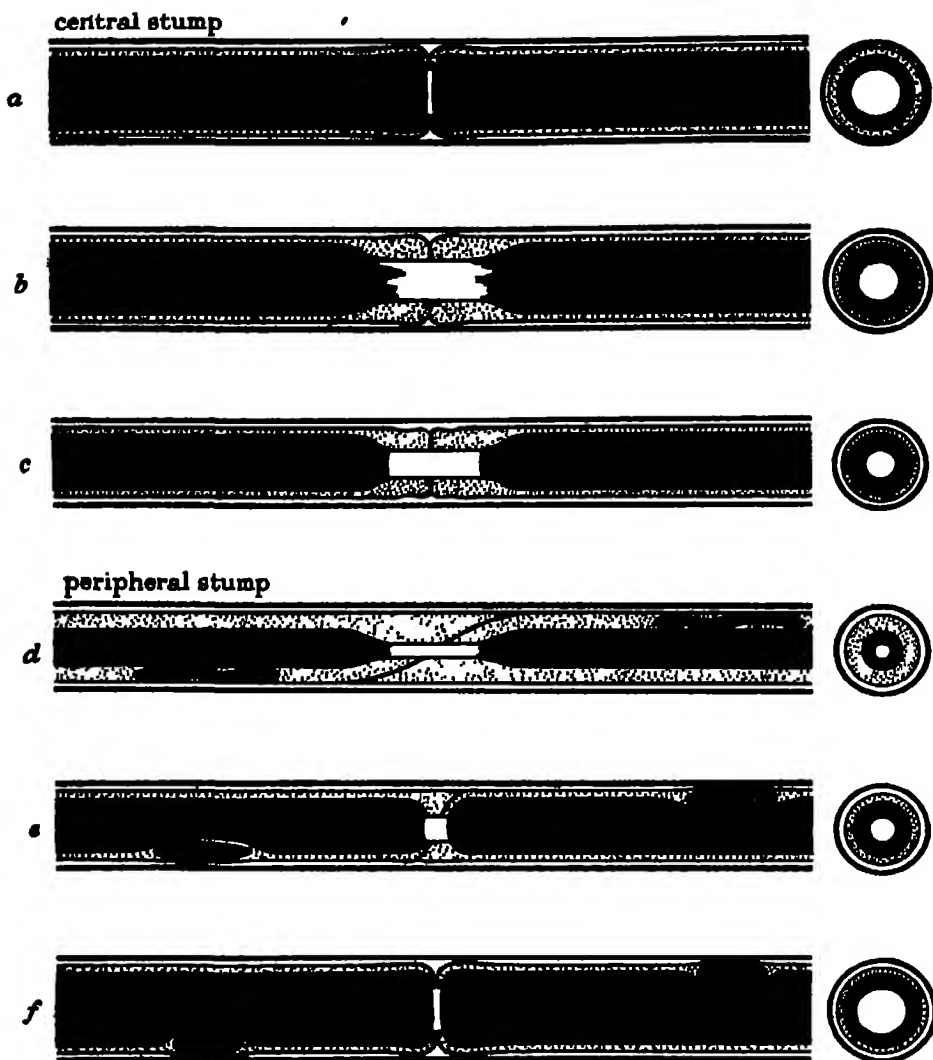
FIGURE 19. Diagrams of comparable nerve fibres at different stages of regeneration, showing the behaviour of the myelin sheath (see text). *a, Normal fibre.* *b, Central stump fibre, 60 days.* The axon has become smaller, while the myelin sheath has thickened and retracted at the node. The neurilemmal tube remains the same size as in the normal fibre. *c, Central stump fibre, 100 days.* The axon has become smaller still: the myelin has the same thickness as at 60 days, and there is still a gap at the node; the whole tube has decreased in diameter. *d, Peripheral stump fibre, 60 days.* The tube is smaller than normally. The axon is very small, and, together with its thick myelin sheath, does not fill the tube. There is a gap at the node. *e, Peripheral stump fibre, 200 days.* Axon and myelin have increased in diameter so as to fill the tube; the myelin has been pressed out towards the node; the tube remains smaller than normally. *f, Peripheral stump fibre, 300 days.* Further expansion of the fibre has restored the normal diameter of the tube, and the thickness of the myelin has been further reduced.

Key to shading, from within outwards: central white core = axon; black ring = myelin; dotted ring = Schwann cell protoplasm; outer white ring = neurilemma; outer black line = endoneurium.

There is a node of Ranvier at the centre of each figure, and figures *d, e* and *f* show Schwann nuclei accompanying the internodal segments.

fibre. This in itself would be an effect which would have to be taken into account in any attempt to describe myelination in terms of the various physical forces involved. Until we have further knowledge of these it will not be possible to give a coherent account of the mode of operation and relative importance of the factors concerned in myelination.

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The histophysiology of the alimentary canal of the earthworm *Lumbricus terrestris* Linnaeus

I. The process of extrusion from the intestinal glands, and other features of the intestinal epithelium

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[Plate 18]

The intestinal epithelium of *Lumbricus* consists of ciliated and gland cells The former show striking histological features in the form of intracellular fibrils, pore-rings and variation (probably phasic) in appearance of the free border The appearance of the epithelium during the expulsion of secretion is described, a process involving co-ordinated changes in both glands and ciliated cells This process (referred to as extrusion) varies, and depending on the speed at which it proceeds, the two categories of restricted and unrestricted extrusion are defined. In both types ciliated cells assist glands in extrusion, but under certain conditions the events in both kinds of cell may occur independently

It is argued that contraction of the intracellular fibrils would account for all the observed deformations of the ciliated cells during extrusion, and the suggestion that the contractility of the ciliated cells is vested in their fibrils is supported by some other evidence With this as a nucleus, a suggested mechanism of extrusion is described The significance of the variable behaviour of the glands during extrusion is considered in relation to existing theories of extrusion, and the classification of glands into holocrine and merocrine.

INTRODUCTION

Recently it was shown that the gland cells in the intestinal lining of *Lumbricus*, produce a proteolytic juice, and in response to the stimulation of secretory nerves, many lose a great part of their substance within 30 min. (Millott 1944). The intestinal epithelium is composed of ciliated and glandular cells, each of the latter being surrounded by a sheath (figure 1) composed commonly of four or five of the former. It is subject to phasic change (see p. 366), its characters, especially those of the free border, undergoing alteration under varying physiological conditions. In certain of these phases, the free tips of the gland cells are completely over-arched by the free ends of the ciliated cells, which thus form a continuous cover over them This was noted by Greenwood (1892) who, in addition, observed that at certain phases the covering border was penetrated by inconspicuous openings. It is natural to suspect that the liberation of secretion is correlated with changes in the free ends of the ciliated cells, and may therefore take place during certain of these phases only. Both Greenwood (1892) and Gurwitsch (1901) hint strongly at the idea, but their evidence is insufficient It is the object of this account to examine the question of the discharge of secretion in the light of new evidence.

TERMINOLOGY

A survey of literature reveals divergence and confusion in the use of the word 'secretion'. It has been used by the majority of workers not only to denote both the process and its product, but also in a varying sense when applied to the former.

[358]

Some use the term widely to cover both the formation of substances within a gland cell and their subsequent discharge, others use it in a more restricted sense, to imply either of these events, frequently without reference to previous work. It is evident that standardization is needed.
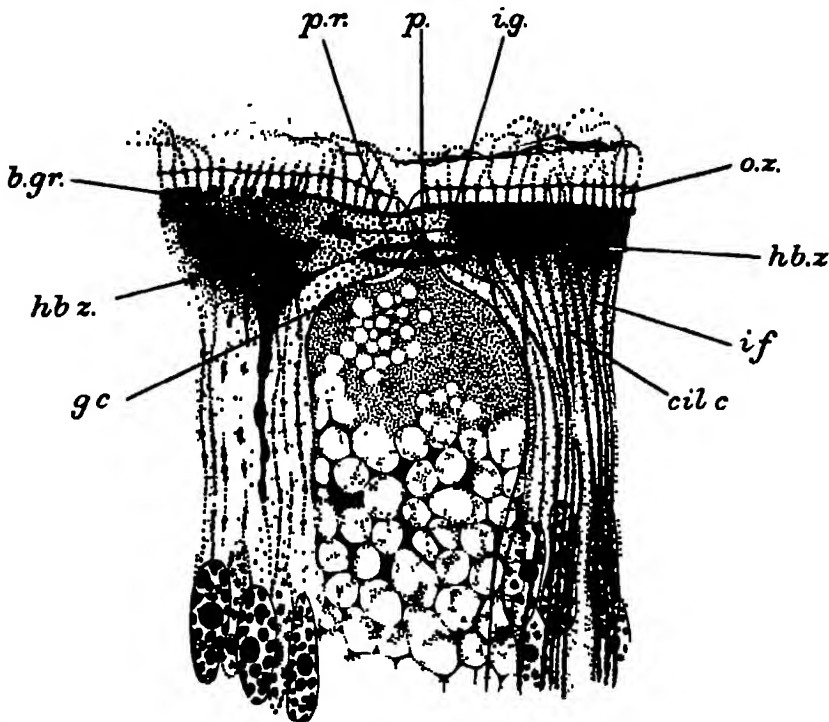

FIGURE 1. Upper portion of a region of the intestinal epithelium of *Lumbricus* (× 1750), from a preparation fixed in Goodrich's modification of Bouin's fluid, stained in Heidenhain's iron-haematoxylin. Worm was fixed 2 hr. after placing in dry corn meal, following 3 days starvation. The lower right-hand margin of the gland cell is seen by transparency through a slightly overlapping portion of the adjoining ciliated cell. *b.gr.* basal granules; *cil.c.* ciliated cell, *g.c.* gland cell, *hb.z.* hypobasal zone; *i.f.* intracellular fibril; *i.g.* inner granules; *o.z.* outer zone; *p.* papilla; and *p.r.* pore-ring.

Thus Matthews (1899) uses 'secretion' in a restricted sense for 'the process of discharge from cells of their metabolic products', whilst Bowen (1926a), though also restricting its use, applies it to a complementary event, viz. formation of the precursors of secretion within a gland cell. Conversely, among recent workers, Hirsch (1931), uses the term in a wider sense for a special form of elimination, namely that in which cells eliminate their own products. The actual process of discharge, is distinguished as 'extrusion', and conceived as an integral part of secretion.

Again, 'secretion' has been widely used as synonymous with 'excretion', thus Ranvier (1887) states with regard to mucus glands: '...la sécrétion consiste dans l'élaboration du mucigène et ce que les physiologistes appellent sécrétion n'est qu'un phénomène d'excrétion.' Ranvier has been widely followed by later workers,

especially Bowen (1926a, b), although Renaut (1911), elected to introduce yet another term, viz. 'excrétion exocellulaire'. Others, however, have distinguished between secretion and excretion on the basis of relative usefulness of the substances concerned, 'excretion' being reserved for the elimination of waste, 'secretion', for the elimination of substances which are useful.

It is the discharge of formed products from their formative cells which is studied here, and therefore the equivalent of 'secretion' as used by Matthews, 'excretion' by Ranvier, Bowen, etc., and 'extrusion' by Hirsch. In view of the confusion existing over the use of 'secretion', and the widespread association of 'excretion' specifically with the elimination of waste, I propose to refer to the process as 'extrusion'.

METHODS

The investigation is essentially a combination of observations on living material under experimental conditions, and on sections of fixed material. By such means it has been possible to discover the precise sequence of events in extrusion. Further, it has been possible to avoid the assumptions concerning the direction of the observed changes, which are inherent in much previous work based on the arbitrary arrangement in series of the different appearances of fixed glands. At the same time, the usefulness of sections of fixed material in elucidating minute structures, has been fully exploited, and correlation of the two approaches proved invaluable in segregating fixation artefacts.

Accordingly, the methods employed for observation fall into two categories: (a) for living material, and (b) for fixed material

(a) Living material

Portions of the intestine of earthworms either fresh from the soil or after 1 to 5 days starvation were excised and pieces approximately 1 mm. square were reduced to a thin layer with the minimum amount of teasing. The tissue was examined under a supported coverslip, in frog Ringer diluted with one-third of its volume of distilled water (for the suitability of this fluid see Millott 1943). As a control similar preparations were mounted either in coelomic fluid from the same worm withdrawn from the same region of the coelom as that from which the intestine had been removed, or in blood withdrawn from the hearts of the same worm by means of a fine pipette. Preparations apparently retained their normal condition for several hours, and were therefore allowed to settle down for 30 min. to 1 hr. before examination.

Most preparations were stained supra-vitally by addition of neutral red to the modified Ringer at this stage. A few were stained for mitochondria in 1:10,000 to 1:20,000 solutions of Janus Green B (G. T. Gurr) or Janus Green Vital (E. Gurr) dissolved in modified Ringer. The tissues were examined under $\frac{1}{12}$ in. oil immersion objectives, and if focusing was observed to exert any pressure, the preparation was rejected. When preparations were examined in modified Ringer, it was replaced by fresh fluid at frequent intervals.

(b) *Fixed material*

Whole worms were fixed in Bouin's or Duboscq-Brasil's solutions, small pieces of excised intestine were fixed in the following fluids: saline Flemming without acetic (Young 1935; Gatenby 1937), Champy, Benda, Altmann, Mann, Schridde, Helly (with neutral formol), Zenker, Bouin, and Goodrich's modified Bouin (Goodrich 1919).

To reproduce experimental conditions, many of the excised pieces were bathed in modified Ringer before fixation, or this fluid was injected into an isolated pocket of gut (see Millott 1944) from which the piece to be fixed was subsequently removed.

Worms were fixed during starvation, during feeding, and at varying intervals afterwards.

Paraffin sections were cut at 4 to 10μ in frontal, sagittal and transverse planes, and stained in Heidenhain's iron alum haematoxylin. Where material had been fixed in saline Flemming, Gatenby's method for cell inclusions (Gatenby 1937) was followed.

Since it has been possible to compare the appearance of the intestinal epithelium in fixed material and in large numbers of living preparations, it is justifiable to comment on the pre-eminently faithful fixation of cytoplasm given in all but one respect by saline Flemming without acetic (see p. 378). Champy also gave extremely faithful fixation of cytoplasm, and yet it is remarkable that these two fixatives, alone of those used, occasionally produced an appearance similar to extrusion, which I cannot consider other than an artefact (see p. 378). Benda's method of fixation (Benda 1899, 1901), in combination with iron haematoxylin, gave astonishing prominence to the intracellular fibrils of the ciliated cells (see figure 2, and figures 3 and 4, plate 18), whilst Bouin and Duboscq-Brasil, by giving sharp staining by haematoxylin, proved useful in studying details of the fibrillar system.

THE STRUCTURE OF THE INTESTINAL LINING

The general histological features of the intestinal lining have already been described in fixed material from various lumbricids, by several authors (see Millott 1944), whose works may be consulted for a general description, but as the process of extrusion in *Lumbricus* has been found to involve the whole epithelium, rather than the gland cells alone, it is necessary to consider in detail certain features having special significance, some of which have been elucidated for the first time.

The ciliated cells

The epithelial border is usually formed by the free ends of these cells over-arching the gland cells which they can completely enclose (figure 1)   In fixed material the free end of each ciliated cell, is sometimes differentiated into three distinct zones, which, as a result of the similar differentiation of their neighbours, form three more or less regular and apparently continuous bands around the epithelial border.

The outer zone (*o.z.* in figures 1 and 2), called by Greenwood the 'basal band', and by Dequal (1910), in the corresponding cells of *Octoclasium*, the 'orlo a spazzola', is also distinguished during life, stains lightly in iron haematoxylin, and is conspicuous in fixed material by its vertical striations which though corresponding in

position to the overlying cilia may persist when the cilia have disappeared (see p. 366). The underlying middle zone, again distinguishable in life, is formed of a layer of basal granules (figure 1, *b.gr.*). It is questionable whether the outer zone is to, be regarded as part of the cell, such structures have frequently been interpreted as cuticular formations (see opposing views of Maximov (1935), and Vignon (1901), and the critical summary of Newell & Baxter (1936).
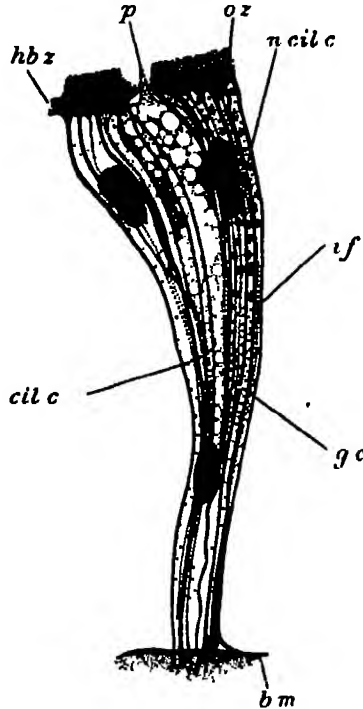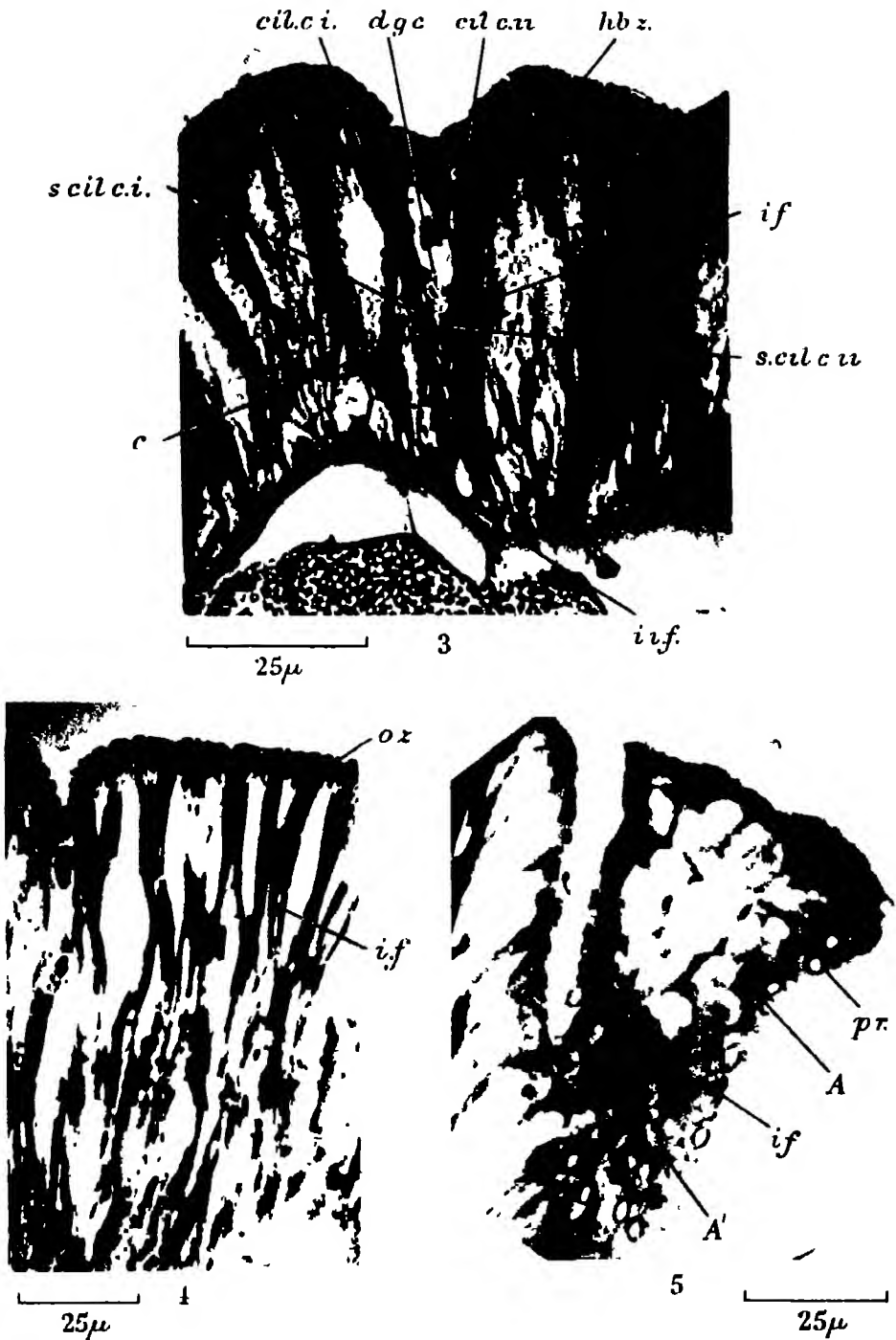

FIGURE 2. Portion of intestinal epithelium of *Lumbricus* (× 1160), showing a gland cell (*g.c.*) inside a sheath of ciliated cells (*cil.c.*). Fixed Benda stained to show intracellular fibrillae (*i.f.*) with Heidenhain's iron-haematoxylin. *b.m.* basement membrane, *n.cil.c.* nucleus of ciliated cell. Other letters as in figure 1.

Beneath the basal granules is the third, and inner zone (*hb.z.* figures 1, 2 and 3), distinguished in previous accounts of ciliated cells by a variety of names, such as 'hyaline zone' (Greenwood 1892), 'angrenzende Zone' (Gurwitsch 1901), 'transparent zone' (Saguchi 1917), 'sub-cuticular zone' (Grave & Schmitt 1925), and 'hypobasal zone' (Kindred 1927). In *Lumbricus* this zone is distinguishable only in sections, varies greatly in appearance, distinctness and extent (1 to 12μ deep), and stains either faintly, so as scarcely to be distinguished, or uniformly and intensely, so as to be distinguished easily (figure 1, right), or in a variegated fashion (figure 1, left), often so pronounced as to give the free border the appearance of an intricate lace pattern. As its appearance is not sufficiently constant to justify the choice of any of the purely descriptive names previously given, it is proposed to retain the term 'hypobasal zone'. Such a choice avoids the assumption implicit in the use of 'subcuticular zone'.

cil.c.i.   *d.g.c*   *cil.c.ii*   *hb.z.*

s.cil.c.i.

if

s.cil.c.ii

c

3

i.i.f.

25μ

o.z

i.f

25μ

4

p.r.

A

if

A'

5

25μ

Beneath the hypobasal zone, the only histological feature significant in this account, is the presence of intracellular fibrils (figures 1, 2, 3 and 4, *i.f.*), which are visible in both living and fixed material, and in the latter they may be traced through the cytoplasm from hypobasal zone to basement membrane, on which they are inserted by ends which are occasionally distinctly forked (figure 2). In sections they range in form from compactly fibrillar to moniliform or granular (see figure 1, and compare also Englemann 1880; Van Beneden, Heidenhain 1911), and in prominence from inconspicuous to dominant, even in the same preparation. After fixation by Benda's method, they attain great prominence (figures 2, 3 and 4). The variation is due to a varying affinity for iron haematoxylin, but not entirely so, for fibrils may attain a certain definiteness and yet remain unstained.

In fixed preparations when the hypobasal zone is shallow, certain of the fibrils can be traced through it into close association with the basal granules and cilia, but when this zone is conspicuous and stains intensely it is not possible to trace them further than the boundary between the hypobasal zone and general cytoplasm. The close relationship between fibrils and basal granules, confirms the earlier observations of Rio-Hortega (1917) on the same cells of *Lumbricus agricola* (= *terrestris*?).

The most remarkable feature of the intracellular fibrillar apparatus, however, is frequently seen in the region where the ciliated cells arch over a gland cell, for here a well-defined ring (figures 1 and 5, *p.r.*) loops through the expanded free end, binding several ciliated cells together by their contiguous portions. Certain of the fibrils situated in the part of the cells abutting on the enclosed gland join the ring and so through it are continuous from cell to cell. This ring, which I propose to call the 'pore-ring' (for reasons that will be obvious later), was originally described by Gurwitsch (1901) and later by Schneider (1908), though both give but a brief description and do not indicate any connexion between the pore-ring and fibrillar apparatus.

The pore-ring varies in diameter from 1 to 10μ, usually stains intensely in iron-haematoxylin, but owing to its situation it is sometimes pressed closely to the neck of the gland cell and hence appears to be a localized thickening of the cell membrane. This may account for the failure of previous authors to make its precise relations clear, but appearances such as those shown in figure 1 leave but little doubt in the matter. Very rarely two rings are present (figure 8), but I could not decide whether they were truly separate, or merely two turns of a close spiral Pore-rings are rarely visible in life, but on two occasions I saw unmistakable indications of them, as judged by their position and relation to the intracellular fibrils.

Concerning their position, I cannot agree with Schneider who states that they are found at the level of the basal granules, for pore-rings are always situated in the hypobasal zone, and despite his contention that they are not always easy to see, after careful examination of fixed material, I have come to the conclusion that they are often missing. It is noteworthy that they are always indistinguishable where the hypobasal zone is inconspicuous, or absent, but the converse does not hold, for they are often invisible when this zone is well developed. This may indicate that a certain degree of development of the zone is necessary for the rings to be formed.

The gland cells

These are very variable in size, form, relative prominence and number of inclusions, as noted by Greenwood (1892). The variation has been described more fully in a previous account (Millott 1944) and is now known to be correlated with the discharge of a proteolytic ferment, for it was there shown that the conspicuous inclusions described by Greenwood, and presumed to be zymogen granules, disappeared from the cells as the proteolytic power of the intestinal juice increased. Greenwood's conjecture concerning their function is therefore confirmed.

The inclusions (described as 'granules' from their appearance in fixed material) are enclosed in vacuoles (see also Greenwood 1892; Schneider 1908; Millott 1944), and obviously correspond to the similarly named bodies seen in many other gland cells (see Renaut, 1911). Their reaction to neutral red *in vivo* is noteworthy, for they stain either homogeneously, red, yellow, or intermediate colours (figure 6, *hom.v.*), or heterogeneously (figure 6, *het.v.*), some parts staining more intensely than others, or even in different colours.

It is significant that the walls of the vacuoles may stain very differently from the inclusions, a feature reminiscent of Renaut's 'lipocrine secretion' (see Renaut 1911), indeed, often the wall reacts alkaline to neutral red, and the contents acid

A remarkable feature of the epithelium is the investment of the glands by a sheath of four or five ciliated cells (noted originally by Willem & Minne 1899). The investment is sometimes complete (figures 6 and 7, 1), but frequently, as also noted by Schneider in the corresponding epithelium of *Eisenia*, the end of the gland nearest the free border is drawn out into a long neck or papilla (*p*, figures 1 and 2), which becomes intercalated between the free ends of the ciliated cells and may even reach the lumen. This happens in the manner described later (p. 368).

Discussion

The most remarkable features of the epithelium are both associated with the ciliated cells, viz. the presence of a complex fibrillar apparatus and associated pore-rings, and the variable appearance of the free border. Concerning the former similar fibrils in epithelial and especially ciliated cells have been described in widely different forms by many workers, but their prominence in *Lumbricus* after Benda fixation is astonishing, and recalls similar structures prominently displayed in these and other epithelial cells by the Achúcarro and Rio-Hortega methods (Rio-Hortega 1917). The classical accounts of these fibrils are those of Englemann (1880), Heidenhain (1911), and Rio-Hortega (1917). Englemann regarded them as continuous with the basal granules, Rio-Hortega confirmed this association, and the former's conception of the fibrils as ciliary rootlets has received wide adoption. Fibrils in the intestinal ciliated cells of earthworms, have previously been described in *Octoclasium* by Dequal (1910), in *Eisenia* by Schneider (1908), in *Lumbricus agricola* by Rio-Hortega (1917), and in unidentified worms by Joseph (1902). Rio-Hortega describes how the fibrils may converge in the central zone of the cell to form a thickened strand ('córdon fibrilar'), as a result of which, since they remain separate at the base and apex of each cell, basal and apical cones

arise. I have not found genuine evidence of Rio-Hortega's 'córdon fibrilar' in *Lumbricus terrestris* and in this my findings agree with those of Joseph and Schneider, although shrinkage due to fixation may bring about a varying degree of approximation of the fibrils in the central zone of the cell (see figure 4). However. apart from this, the fibrils have been found to differ from existing descriptions of such structures both in earthworm and other forms in several respects. Firstly, the connexion between some of the fibrils and the pore-rings has been noted here for the first time; secondly, I cannot confirm the permanent continuity of any of the fibrils and basal granules; and thirdly, in common with previous workers in earthworms, I have not been able to discern any cross-striation in the fibrils like that described by Heidenhain (1911), in certain ciliated cells of salamanders.

The fact that the fibrils appear unmistakably clear in life, is most significant, and agrees with the similar observations made by Vignon (1901), and Grave & Schmitt (1925) on living ciliated cells of the gills of *Mya* and *Lampsilis*, tentacles of *Plumatella*, and the marginal cells of the nephrostome of *Lumbricus*, etc. On the other hand, these observations are in sharp contrast with those of Carter (1924, 1926), who did not see fibrils in the velar cells of either living or fixed nudibranch larvae, and only in fixed material in the cells bearing the lateral cilia of *Mytilus* gills.

Despite their variable form, their constant occurrence and prominence in fixed preparations and especially their appearance in life indicate that the fibrils in *Lumbricus* are not artefacts of fixation. The status of such structures has occasioned endless controversy (for critical review of the earlier work see Wilson 1928, and especially Heidenhain 1911). Butschli (1894) denied the existence of fibrilar structures in general, believing they were formed of the mesh work between lines of alveoli, Kolaĕv (1910) and Saguchi (1917), extending this conception, believed that the fibrils were formed by the deeply staining, longitudinal shafts of a cytoplasmic reticulum, Rabl-Ruckhard (1868) and Leydig (1883), regarded them as folds of the cell membrane In my preparations the clear discreteness of the fibrils in transverse sections of the ciliated cells invalidates such an explanation. Concerning the former view, it is significant that quite apart from the question of whether such a reticulum exists in life, the evidence for it, as expounded by Kolaĕv and quoted by Saguchi, has a damaging weakness, because he states: '...während die Querbalken schwacher ausgebildet sind, infolge dessen sie nicht immer wahrnehmbar ist.' Such an interpretation of the fibrils in the ciliated cells of earthworm intestine cannot be accepted, for not only are no connexions visible between them in fixed preparations, but they are clearly discrete in the living state.

Other workers, notably Benda (see Heidenhain 1911) believed the intracellular fibrils to be formed of mitochondria arranged in rows and the possibility of the intracellular fibrils of the earthworm being composed of such bodies, or at least associated with them, would appear to be supported by their marked affinity for iron-haematoxylin after Benda's formol-chrome fixation. However, such a suggestion is weakened, not only by their inconstant granulation, even after fixation with a mitochondrial fixative such as Schridde's fluid, but also by their persistence and prominence after fixation in fluids containing alcohol and acetic acid, and especially

by their refusal to respond to supra-vital staining in 1 : 10,000 to 1 : 20,000 solutions of Janus Green B or Janus Green Vital.

In the light of more recent researches into fibril structure (Meyer 1929; Meyer & Mark 1930; v. Muralt 1933, Bear, Schmitt & Young 1937; Bernal 1938; Astbury 1947), especially as the intracellular fibrils described here are well differentiated and either contractile or elastic (see p. 374), one would expect them to yield evidence of a linear, or at least orderly, arrangement of some or all of their molecules. Since no attempt has been made to investigate their ultrastructure, this remains nothing more than a possibility, though it is noteworthy that intracellular fibrils in other forms (see Englemann 1880, Schmidt 1931, Schmitt 1939), similar in disposition to those described here, have long been known to give possible indications of an orientated ultrastructure by their birefringence and dichroism.

Concerning the changes in the free border of the epithelium, I cannot, for the moment, do more than confirm generally, the observations of Greenwood (1892), Gurwitsch (1901) and Joseph (1902) in relation to the nature and extent of the variation. Although their views diverge in detail, they agree that the free border can lose its cilia and basal granules. I have observed the variations they describe in both living and fixed material, and in the same preparation or section, ciliation may be well developed, sparse, or completely lacking, basal granules may be clearly defined or indistinguishable, the outer zone may be hyaline, sparsely, or densely striated, and the hypobasal zone, deep, shallow, or even indistinguishable.

The changes described by Gurwitsch are more extensive than those noted by the other two, for he mentions the ultimate disorganization of the outer zone: 'Der Stäbchensaum ist nun ganz niedrig und unregelmässig, der Flimmerbesatz bis auf einen relativ spärlichen Rest reducirt; . . .' I have frequently seen this, but in some sections of fixed material the outer, middle, and part of the hypobasal, zones had disappeared completely, leaving the papillae of the gland cells, the pore-rings, and part of the hypobasal layer, exposed to the lumen. In view of its occurrence in fixed material only, it is tempting to ascribe this phenomenon to bad fixation, but such a view is questionable, for not only was the fixation generally faithful, and the affected cells well fixed in other respects, but regions of the epithelium alongside, showed a complete and unmutilated border. It is further significant, that what appeared to be progressive stages in disorganization of the border could be picked out in many sections. Nevertheless, it is emphasized, that I have not yet seen such a degree of change in living preparations under experimental conditions.

Whether the changes are truly phasic as suggested by Greenwood, or not, must remain an open question, for it is significant that no observer has claimed to see the changes actually occurring in life, and it is noteworthy that all the changes described hitherto are conjectural, and based on the arrangement in series of different appearances in both living and fixed preparations, chiefly the latter. Greenwood conjectured that the cilia were retractile, and Joseph, whilst admitting he had not observed the outgrowth of cilia, postulated that they sprouted from the basal granules, passing through rodlets in the outer zone.

The variation of the free border is unquestionable, and unless it is assumed that the intestinal epithelium of the earthworm contains many types of cell, identical

but for the characters of their free border, which grade almost imperceptibly into one another, an explanation such as that advanced by Greenwood is difficult to avoid. It is possible that the ciliated cells degenerate and are continually replaced, but this raises the question of replacing cells, for it is certain that they would have to appear in large numbers. Replacing cells have been described by Vejdovsky (1905), Joseph mentions 'Ersatz-zellen' lying deeply in the epithelium, but Schneider accepts them only with reserve, Greenwood could not establish their constant occurrence, and their presence is doubted by Sterling (1908). They are not mentioned by any other author, and I have failed to find any trace of them.

A comparable variation in the structure of epithelial borders has been recorded in other forms, notably *Hydra* (Greenwood 1888), *Teredo* (Potts 1923), and especially *Jorunna* (Millott 1937).

Finally Gurwitsch makes the most interesting observation that the changes he describes in the free border of the ciliated cells, go hand in hand with the extrusion of secretion from the gland cells 'Die Drüsenzellen entleeren sich nun allmählich vollständig und Hand in Hand damit geht auch eine weitere Umwandlung der Flimmerzellen vor sich....'

Such an observation, when correlated with my own on fixed material, appears, at first sight, to afford a completely satisfying explanation of the disorganization of the free border, and of the way in which secretion formed by the gland cells finds its way out, for the gland cells exposed by the disorganization of the border, would be able to extrude their secretion freely into the lumen. Just how far this is valid, may be gauged from an observation of the actual process of extrusion.

THE PROCESS OF EXTRUSION

The sequence of events

When preparations of living intestinal epithelium are observed in modified Ringer's solution a number of gland cells begin to extrude. In many instances, the process starts immediately on mounting the preparation, no doubt as a result of the unavoidable mechanical stimulation, since the gland cells have already been shown to be excitable and to respond readily to nerve stimulation (Millott 1944). In preparations which have been given time to settle down (see p 360), from time to time gland cells will start to extrude, without any obvious external stimulus, especially when they are heavily laden with zymogen vacuoles. It is proposed to begin by describing the events observed in two instances in small portions of intestinal epithelium mounted in modified Ringer and stained in neutral red. Later, the significant variations encountered during the course of numerous observations, will be described.

The sequence of events in the first instance, illustrated in figure 6 is as follows:

Stage I

This immediately follows the condition of apparent rest in which the gland is completely enclosed by the ciliated cells, the outer zone of the epithelial border is continuous, and there is no trace of any channel or pore between the contiguous tips

of the ciliated cells. At first the tip of the gland begins to swell, due partly at least to the transference of cytoplasm from the middle zone, which is progressively constricted as the ciliated cells begin to bulge into it.

Stage II

The rounded tip of the gland grows out towards the lumen as a small papilla (*p.*), which is insinuated between the tips of the ciliated cells, and appearing to force them apart, eventually bulges to the exterior through a small gap in the free border, as a clear rounded mass of cytoplasm. At the same time, a number of zymogen vacuoles ('granules') near the tip begin to swell, their contents becoming more translucent as they increase in size. Simultaneously, changes are observed in the ciliated cells; they begin to shorten, more markedly at first in the middle of their free ends, so that a shallow invagination (*inv.*) arises.

Stage III

As the papilla moves outwards between the tips of the ciliated cells, by now widely separated, the gland cell gradually changes shape and shrinks, not evenly but rather abruptly at about the middle of its height. The ciliated cells appear to 'elbow' into its sides at this level, forming a constriction, and at the same time the invagination of their border becomes more pronounced.

Stage IV

Zymogen vacuoles now begin to move into the papilla, which rapidly changes form as it increases in size, and it is evident that the whole upper region of the gland is beginning to flow out. The invagination of the ciliated cells deepens as they continue to shorten and change shape, pushing further into the middle of the gland and increasing the constriction. A few of their cilia, hitherto all very active, begin to beat more feebly, especially those alongside the papilla where they tend to adhere to its cytoplasmic envelope.

Stage V

The whole cytoplasm and contained vacuoles of the upper half of the cell appear to be squeezed to the exterior through the wide gap between the tips of the ciliated cells and the extruded cytoplasm and contained vacuoles show signs of swelling and dispersion, following breakdown of the envelope (*e.*). The cytoplasm of the basal portion, which is free from zymogen vacuoles and usually contains the nucleus, rounds off, separating from the portion being extruded by a newly formed membrane (*m.*).

Meanwhile the ciliated cells are still shortening, the invagination appears like a deep fissure lined by cilia, which gradually become stationary and flaccid. The outer zone and basal granules are no longer distinguishable along the invaginated border.

Stage VI

The cytoplasm outside the newly formed membrane undergoes complete dissolution, liberating the contents of the vacuoles, which swell rapidly, their outer zones at first becoming progressively fainter, while the diminishing centre remains relatively dense and conspicuous, until finally they disappear. Masses of such contents extruded from glands nearby collect along the border of the epithelium.

The ciliated cells then begin to return to their normal form and height, the invagination gradually disappearing; their ciliation is sparse, many cilia lying flaccid against the border, the remainder beating feebly. No signs of a return to the highly motile border was observed, although the preparation remained
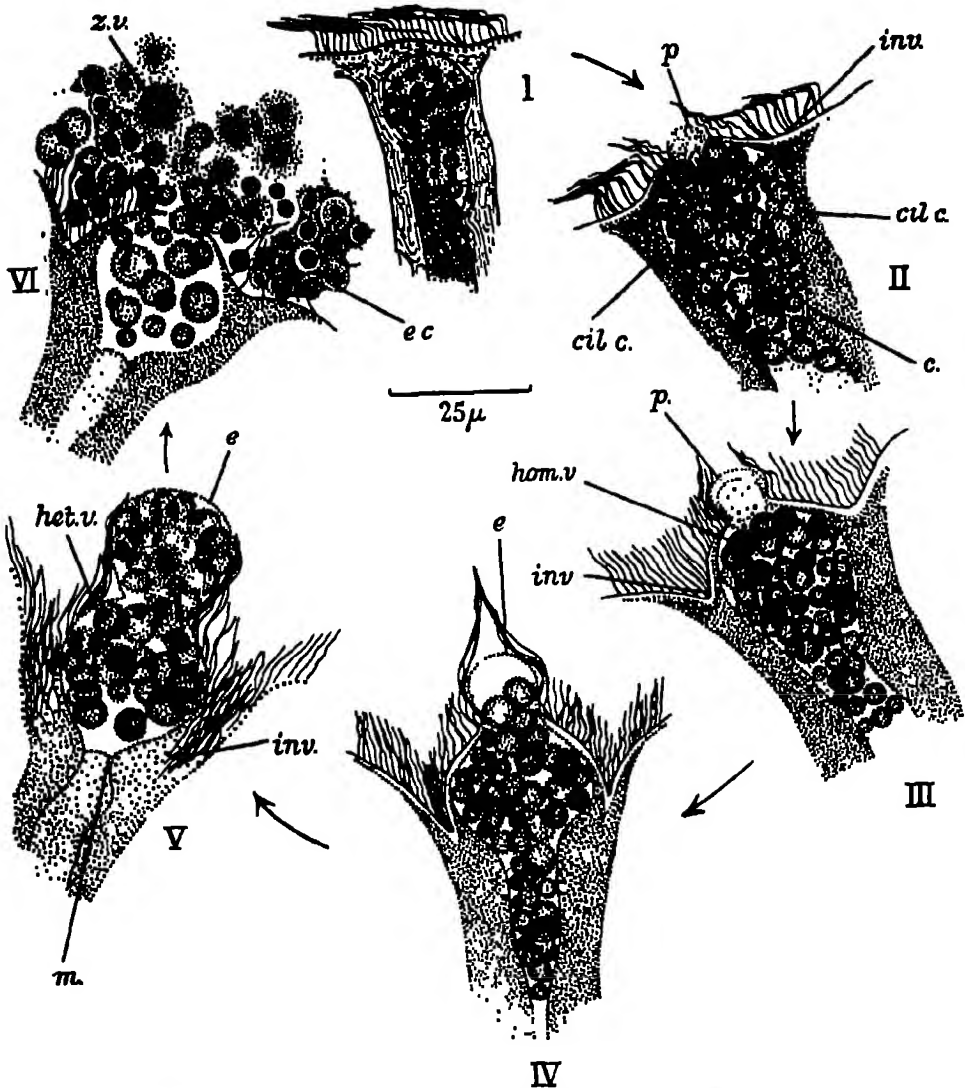

FIGURE 6. Unrestricted extrusion illustrated by successive events seen in the upper regions of a living gland cell and two adjoining ciliated cells, arranged as stages I to VI. Drawn from a preparation of the intestinal wall of *Lumbricus*, mounted in modified Ringer's solution and stained in neutral red. Worm used was taken directly from soil. *c.* constriction in gland cell formed by localized swelling of ciliated cell; *e.* envelope of extruded cytoplasm; *e.c.* cytoplasm and contained zymogen vacuoles extruded from neighbouring gland; *het.v.* zymogen vacuole with deeply staining centre; *hom.v.* uniformly staining zymogen vacuole; *m.* membrane of residual cytoplasm of gland cell; *z.v.* discharged zymogen vacuoles swelling and undergoing dissolution. Other letters as in previous figures.

apparently healthy for $1\frac{1}{2}$ hr. In the preparation figured, extrusion was complete in 30 min., every stage passing gradually into the next, and it was therefore possible to time the various events by stop-watch. By timing five such sequences, in different preparations, it was possible to gain some idea of the extent of variation. It was found that at room temperature, with a pH of the contents of the freshly excised gut (estimated by phenol red against a colorimetric standard), varying between 7·2 and 8·1, the time of attaining the three stages, beginning of invagination of the border, reaching of the exterior by the papilla, and the completion of extrusion, varied between the respective limits 4 to 5, 17 to 20, and 27 to 34 min.

The above sequence of events is typical of some instances only, in others, extrusion occurred rather differently. A second process may be followed from figure 7, by reference to the middle gland. Broadly speaking, its behaviour is similar to that already described, but stage II and following stages show some significant differences. Firstly, the extruded cytoplasm appears as a clear, spherical droplet, devoid of zymogen vacuoles. Secondly, the tips of the ciliated cells do not move far apart, and the droplet arises from a papilla passing through a relatively narrow gap. Thus, instead of disappearing quickly, the papilla persists, and forms a progressively attenuating column, as its expanded outer end is pushed farther away from the epithelium, on the tips of the surrounding cilia. Eventually, the column, stretched to the breaking point, ruptures, and liberates a clear vesicle (stage Va) which swells, disintegrates, and sets free its contents.

A further difference is that only the cytoplasm at the extreme tip of the gland cell is extruded. The most significant difference, however, is in the timing. Up to the stage of the papilla reaching the surface, the events proceed with much the same speed as that noted above, and moreover with little variation, but the elongation and rupture of the papilla, and separation of the vesicle, may take from $\frac{1}{2}$ to $\frac{3}{4}$ hr. after stage IV has been attained.

Taking the last two differences in conjunction, it is clear that the second process is much slower than the first, a fact which is strikingly obvious when the two are viewed side by side. However, when the two series of events are considered in the light of many observations it is evident that apart from the difference in speed at which extrusion is accomplished and the fact that in some cases, the size of the opening between the tips of the ciliated cells is wide, and in others narrow, there is no constant difference between them. So far as all the other differences noted above are concerned, they are obvious only because the two series of events cited, happen to be two extremes, between which all intermediate conditions can be observed. Again, although generally speaking, gland cells lose much more of their substance by the first method, than by the second, the distinction cannot be stressed, as protracted observations on the behaviour of a single gland cell have proved impracticable, and it is possible that the slower process may be repeated several times in succession.

It is most significant, however, that the two constant differences are associated, for the first, relatively rapid process, is always associated with extrusion through a wide opening between the ends of the ciliated cells, and the second slower process, with extrusion through a narrow one. This at once suggests that a deciding factor
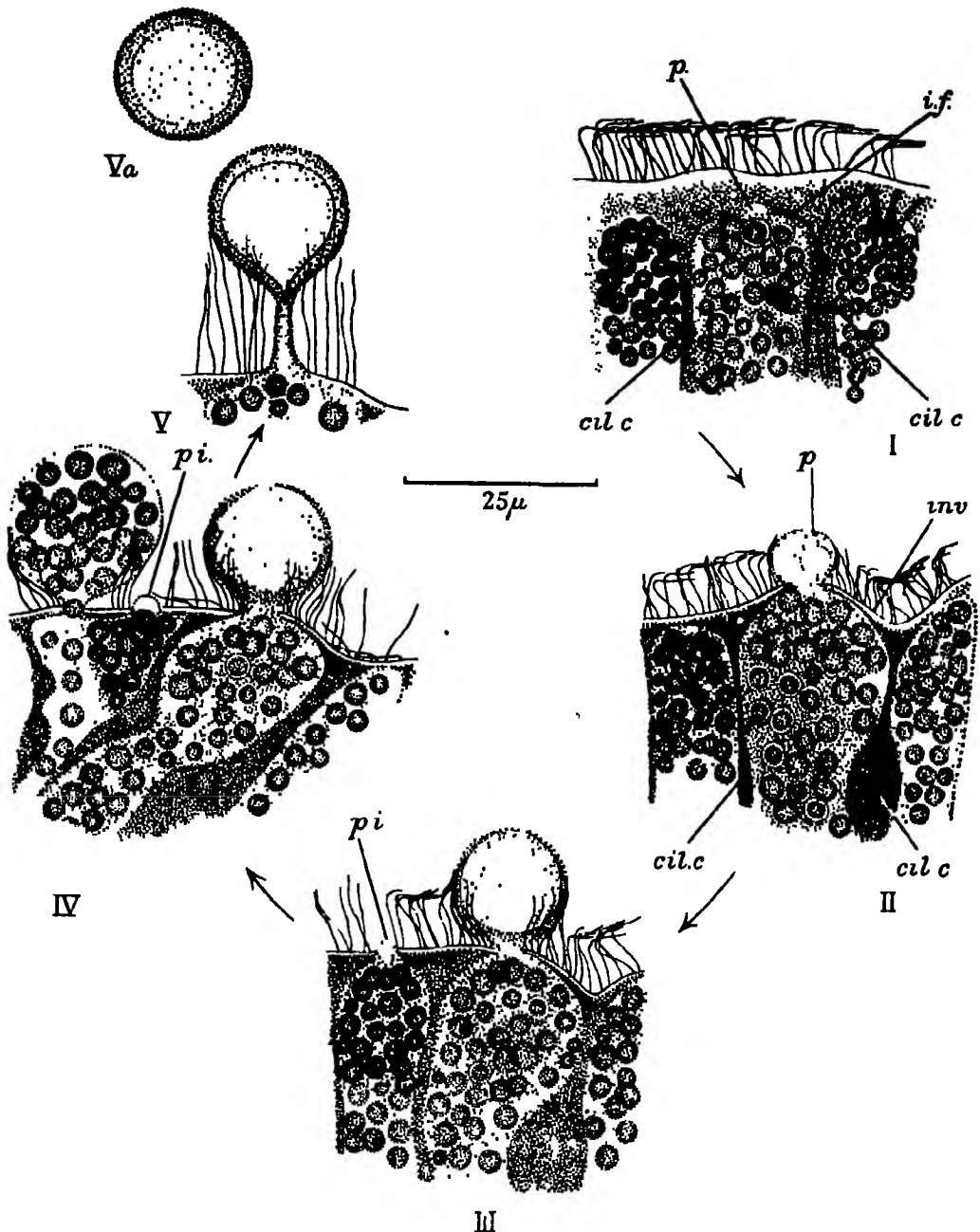
FIGURE 7. Restricted extrusion illustrated by successive stages I to V, drawn from a piece of the living intestinal epithelium of *Lumbricus*, mounted in modified Ringer and stained in neutral red. The upper portions only of the three gland cells and the intervening ciliated cells are shown in stages I to IV. Stage V shows the extreme tip of the centre gland cell only, stage Va shows the vesicle of cytoplasm finally liberated from this cell. Worm had been starved for 5 days when this portion of the intestine was excised. *p.i.* abortive papilla Other letters as in preceding figures.

is associated with the ree border of the epithelium. A study of sections reveals that this factor is probably the presence or absence of the pore-ring for when glands have been fixed in the act of extrusion, clear indications of the first (more rapid) method, are invariably associated with the absence of a pore-ring, those of the second, with its presence. Indeed, even when there is no obvious indication of extrusion in sections, it is significant that when there is a wide gap in the epithelial border over the tip of a gland cell, a pore-ring is always missing. The fact that the converse does not always hold good does not refute this view, since a gap may have been fixed as it was forming, or closing. Thus two types of extrusion can now be distinguished, one, which I propose to designate *restricted extrusion*, occurs where the epithelial border has a restricting influence, the other, which may be termed *unrestricted extrusion*, occurs where the epithelial border has no such influence. The former is associated with the presence of a pore-ring, the latter, with its absence.

It is immaterial whether the preparations are mounted in modified Ringer, blood, or coelomic fluid, for both types may be seen in all these fluids, and moreover there is a constant association of activity in glands and in their sheath of ciliated cells.

In view of the evidence of the limiting power of the pore-ring presented above, it is pertinent to consider whether there is evidence of such power ever being great enough to prevent extrusion altogether. Gurwitsch (1901) merely hints cautiously at the idea of the pore-ring having some significance in extrusion, in saying of it 'Ob das Ganze eine eigenthümliche Vorrichtung fur Excretionszwecke ist, lässt sich mit Sicherheit nicht sagen'

For the moment, it is not possible to answer this question with any degree of certainty, but under experimental conditions, it is certainly possible for events in the epithelial border to get wholly out of step with those in the gland cells As a result, either abortive papillae are produced, which though growing out from the tip of the gland towards the free surface just as in extrusion, are halted in the hypobasal zone and eventually retracted, or conversely, pores are formed in the epithelial border over the tips of the glands, and yet no changes characteristic of extrusion are visible in the latter. Such observations are suggestive, though nothing more, of a system of control vested in the epithelial border, which is wider in scope than the purely flow-restricting function already envisaged

Analysis of the process of extrusion

From the foregoing observations it appears that the ciliated cells take an active part in extrusion, by pressing against the gland cells, constricting them, and forcing out part of their contents. However, it must be emphasized that even if this be true, it is certain that all the changes undergone by the ciliated cells during extrusion are by no means essential to the process, for sometimes gland cells have been seen to extrude, without any significant change in the surrounding ciliated cells, other than a slight swelling of their middle region. It is evident therefore, that there may be an alternative explanation of the observed events, namely, that the glands may be the active agents, and by swelling and consequent change in shape, may effect a complementary but passive change in the ciliated cells.

It now remains to decide between the two alternatives. If the former view is to be maintained it must be shown that the ciliated cells, by changing their form in the way described, can exert a significant pressure on the adjoining gland. That this is so, is indicated when events take a slightly abnormal turn, as in figure 7. Here, the ciliated cell on the right side only of the middle gland cell, exhibited the marked change of form already described. That on the left, merely swelled slightly in the middle. If the view that the ciliated cells exert pressure on the gland cell during extrusion is correct, then obviously greater pressure should be exerted from the right side than from the left. A comparison of stages III and IV clearly substantiates this, for the axis of the gland cell is swung out of the vertical and over to the left. At the same time, the gland cell on the extreme left is grossly distorted, and extrudes abnormally, as shown by the fact that extrusion, though beginning with the production of a papilla (*p.*), ends by the bursting out of the contents from the base of the gland cell owing to rupture of the border, while the apical contents, which are normally extruded, remain inside the cell beneath the abortive papilla. No doubt it is the sudden release of the contents by rupture, which permitted the marked change in orientation.

Further substantiation comes from the fact that it was frequently possible to induce changes which are an exact replica of those seen in the living ciliated cells, by adding 0·05 % NH_4OH to the modified Ringer. Since the response occurred in the cells of the epithelium around spaces left where a gland had either been expelled, or had discharged most of its contents, it is obviously active, and not merely a passive reflexion of certain changes in the glands

These observations at once raise the question as to whether the whole process of extrusion occurs by the action of the ciliated cells. Similar experiments to the foregoing indicate that this is not so, for changes characteristic of extrusion can be induced in ciliated cells, and yet their accompanying gland cells, even though loaded with zymogen vacuoles, and provided with a passage through which to extrude owing to changes in the epithelial border (see below), do not extrude at all Such experiments show that some particular physiological state of the gland cells must be attained before extrusion can occur, and afford striking support for the views of Hirsch (1931) concerning restitution

The significance of the intracellular fibrils and pore-ring

From the foregoing evidence it is clear that extrusion from the intestinal glands may involve co-ordinated activity in both gland cells and ciliated cells, and in view of the well-defined changes in shape that occur in the latter, they must be endowed with marked contractility. It is therefore pertinent to seek evidence of appropriate structural differentiation in the ciliated cells, and in view of what has already been described of their structure, the intracellular fibrils at once come to mind. The idea agrees, not only with their remarkable degree of development and prominence, but is supported by other evidence from both living and fixed tissues. Firstly, it is significant that the fibrils are most prominent in living preparations, during the early stages of extrusion, when the change in form of the ciliated cells is just

beginning (see figure 7, *i.f.*). Indeed, the fibrils were not observed until extrusion commenced, and later on, as the changes in form of the ciliated cells became greater, they disappeared from view (figure 7 stages II *et seq.*). At the stage of greatest prominence, they often stand out very clearly where they fan out over the tips of the glands, and in optical section, appear as a row of bright dots.

Secondly, when the fibrils remain visible throughout the process of extrusion, they may be seen to shorten with the ciliated cells, always pursuing a more or less
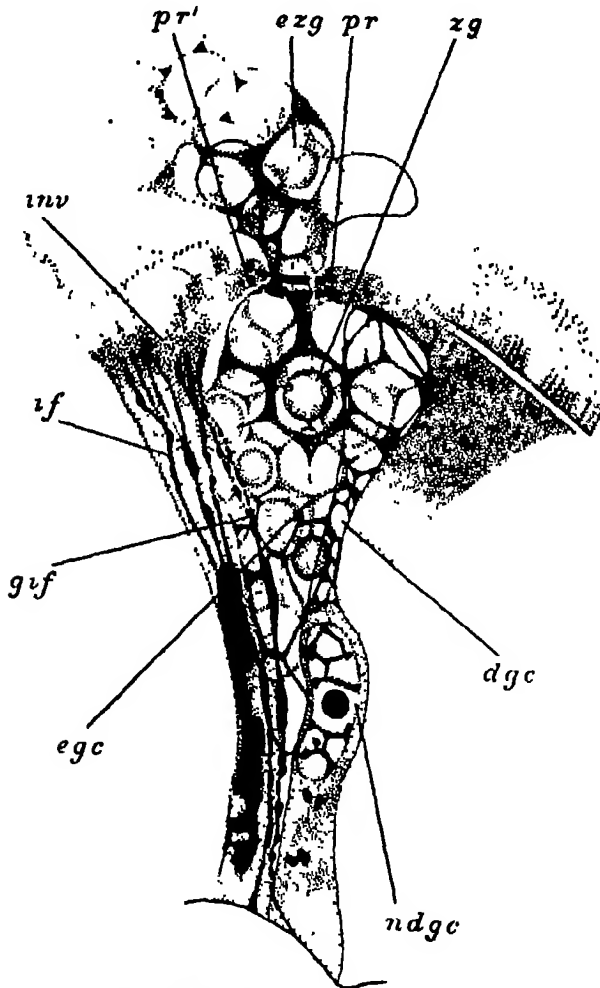
FIGURE 8. Portion of the intestinal epithelium of a young earthworm, with a gland cell fixed in the act of restricted extrusion. Fixed in saline Flemming-without-acetic (Young's modification), stained Heidenhain's iron-haematoxylin (× 3250). The worm was fixed after being placed for 4 hr. in a slightly moistened mixture of agar and corn flour, following a period of 5 days' starvation. *Note.* The flanks of the extruding gland are seen by transparency through the over-lying ciliated cell (on the left), and discharged gland cell (on the right). *d.g.c.* discharged gland cell; *e.g.c.* extruding gland cell; *e.z.g.* zymogen 'granule' in vacuole of extruded cytoplasm; *g i.f.* intracellular fibril appearing granular, *n.d.g.c.* nucleus of discharged gland cell; *p.r., p.r.',* parts of double (?) pore-ring seen in optical section; *z.g.* zymogen 'granule' within vacuole. Other letters as in preceding figures.

straight course, and never becoming markedly sinuous. The fibrils are therefore unquestionably either contractile or elastic.

Finally, although their contractility has not been demonstrated directly, the fact that owing to their disposition (see p. 363), their imagined contraction could bring about the changes in shape observed in the ciliated cells, constitutes valuable indirect evidence for such a property. Thus not only would their contraction diminish the height of the cell generally, but the free end of the cell, being supported
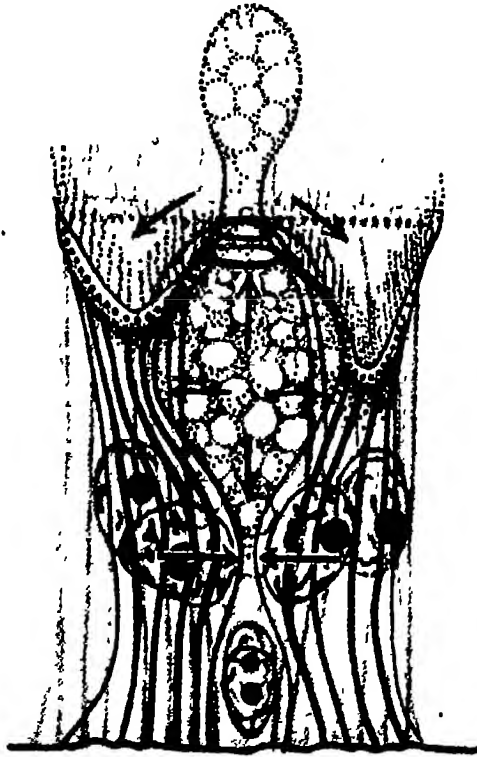
FIGURE 9. Diagram illustrating the mechanism of extrusion (see p. 376). The features and disposition of a gland and two adjoining ciliated cells during extrusion, are indicated by heavy outlines, those of the same cells during the state of apparent rest, by faint outlines. The arrows indicate stresses set up during extrusion (see p. 376).

on either side by adjoining glands or ciliated cells, would not be pulled down by the fibrils so readily at the sides as in the centre, which is relatively unsupported. This would account for the invagination actually observed.

Now that the meaning of the changes in form of the ciliated cells has been interpreted, it is easy to advance an explanation of how the pores through which extrusion occurs are formed, at the same time clarifying the possible significance of the intracellular fibrils and pore-ring, and accounting for their structural relationships. The observed invagination of the free end of the ciliated cells, in the first place, will subject the free border of the epithelium to a lateral stretch (figure 9). The tension will probably be increased by swelling of the tips of the glands beneath

(see p. 367), and since a component of the force they exert will act vertically, the point of maximum strain will be in the region where the ciliated cells are contiguous as they arch over the gland cell. It is not surprising, therefore, that it is precisely in this region, that the continuity of the border is seen to be broken, the ciliated cells parting along their line of junction, forming a channel through which the cytoplasm of the gland cell tip is pushed out as a papilla.

From such an inference it follows that the pore-ring is centred in the region of maximum strain, added to which it is obviously in the most effective position possible for limiting the size of pore, and rate of outflow. Further, the continuity of the pore-ring and intracellular fibrils is similarly explained, for the attachment of one end of the latter to the basement membrane, would provide an anchoring support for the pore-ring against the postulated up-thrust exerted by the gland cell during extrusion, as a result of its own activities, or those of the ciliated cells, or both.

Summary of evidence concerning extrusion

From the foregoing it is clear that extrusion from the intestinal glands involves two series of events, firstly those in the glands, and secondly those in the surrounding ciliated cells. The two are complementary, and normally proceed in harmony, but they are not mutually inter-dependent, for under experimental conditions the two may 'get out of step' to a varying degree, and one series of events may even occur without the other, usually to the detriment of the process as a whole, which may, as a result, be abortive.

So far as the gland cells are concerned, extrusion consists of the discharge of a variable portion of their cytoplasm and its contents, through a channel between the ciliated cells, when a particular stage of their life has been reached. In this, they are assisted by the ciliated cells, the function of which in extrusion appears to be threefold, first to assist by exerting pressure on the glands, secondly to aid formation of the channels in the epithelial border by invaginating and thus exerting a force tending to pull apart their free ends, and thirdly, in restricted extrusion, to limit the rate of discharge. These functions seem to be vested in their powers of contraction, their intracellular fibrils, and their pore-rings. The mechanism is diagrammatically summarized in figure 9.

DISCUSSION

Several conjectures, but no observations, have previously been made concerning the process of discharge of secretion from the intestinal glands of earthworms. Greenwood's conjectures that the secretion is discharged through channels in between the ciliated cells of the intestinal epithelium, and that the glands varied in their rates of discharge are fully substantiated. On the other hand, certain ideas that have been advanced concerning the relation between extrusion and the supposed phasic changes in the epithelial border cannot be substantiated. Thus the statement made by Gurwitsch, quoted on p 367, concerning the concomitant changes during extrusion in the glands and in the free border of the epithelium, is not confirmed. Neither is Greenwood's statement that the extent of ciliation of the epithelium varies directly with the number of secretion granules in the glands.

Again, there is no evidence that the glands extrude simultaneously as indicated by Schneider's conjecture: 'Diese muss sich ziemlich gleichzeitig bei allen Zellen abspielen, da häufig ganz allgemein die Zellen vollig sekretleer sind und dann sehr dünn erscheinen.'

Nevertheless, structural changes in the border do occur in life, for, as stated on p. 368, the number of active cilia may be considerably reduced, the basal granules and outer zone may become indistinguishable. It is emphasized, however, that these appearances must be interpreted with great caution, as they do not necessarily represent a true change in structure. Thus the reduction in ciliation is in many cases only apparent, due to the cilia ceasing to beat, becoming flaccid, and lying closely applied to the surface of the epithelium, where they can be detected only with difficulty. If, however, the saline in which the preparation is mounted, be replaced by fresh fluid containing 0 05 % ammonium hydroxide, the flaccid cilia rise up in quick succession, as the fluid passes over the epithelium, resume their beat with great vigour for a minute or so, and then return to the flaccid state. Again, when the invagination of the free border of the ciliated cells occurs during extrusion, they are considerably distorted, and the observation of details in the border is not easy. Under such conditions, it is clearly dangerous to attribute the invisibility of features so minute, to a change in structure

At this point it is appropriate to consider critically the validity of the observations recorded in this account. Three main objections have been raised against accepting appearances such as those previously described, as due to extrusion. Firstly, they have been held to be the result of osmotic disturbances, due either to fixation, or to the use of fluids other than the animal's own tissue fluids for mounting living preparations (Vignon 1899, Petersen 1912, de Boissezon 1930) Secondly, they have been ascribed to the compression of living tissues and thirdly, to traumatic shock following removal of the tissue for examination (Vignon 1899).

Considering these objections in order as applicable to the preceding observations, if the first is to be allowed, then since the appearances of extrusion in living and fixed preparations of *Lumbricus* agree so closely, it must be assumed that they are the result of osmotic disturbances in either instance. Such a conjecture would agree with Vignon's observation that 'phosphate de soude', 'chlorure de sodium' and 'solution de sucre' etc, in concentrations described as 'indifférentes', when placed in contact with the epithelium lining the gut of *Chironomus* larvae, produced appearances similar to those attributed by many workers to extrusion. Again, Wigglesworth's observation (Wigglesworth 1931), that osmotic disturbances due to fixation, or immersion in saline, produced in the malpighian tubes of *Rhodnius*, artefacts similar in appearance to what has many times been described as extrusion, offers more justifiable grounds for the contention

In earthworm, however, since the appearances interpreted as extrusion can be seen in excised tissues, not only in modified Ringer, but also in blood and coelomic fluid taken from the same worm as the tissue, it is unlikely that they are due to osmotic disturbance.

The objection on grounds of possible effects of mechanical pressure is less easy to refute, especially since Greenwood (1892) has described the production by com-

pression, of bladders from the intestinal epithelium of *Lumbricus*. However, in view of the precautions taken to obviate the effects of mechanical pressure (p. 360), it is not considered to be a significant factor, and moreover it is noteworthy that the bladders mentioned by Greenwood are not described or figured as having any relation whatsoever to the glands. Quite apart from this, however, it is questionable whether an objection on the grounds of an imaginary compression could be sustained in any event, for the preceding account has shown that pressure generated within the living epithelium may be an integral factor in extrusion. Even therefore if the bladders produced by compression in the intestinal epithelium of earthworm, noted by Greenwood, were produced from glands, it is not justifiable, on these grounds alone, to reject them as compression artefacts having no relation to normal extrusion.

With reference to the third objection, it is not practicable in living preparations to eliminate the possible effects of traumatic shock, for all attempts at observing the intestinal epithelium *in situ* proved unsatisfactory. No such objection could be raised, however, against the extrusion observed in sections of earthworms that had been fixed whole.

Finally, the generally uniform appearance of extrusion in *Lumbricus*, obtained after using a wide variety of fixatives under varying physiological conditions, its close correspondence with that of extrusion seen in living preparations, and, above all, the presence in the epithelium of well-defined structures so strikingly adapted for participation in the process described, can leave but little doubt that it is a normal function and not a mere artefact.

Undoubted artefacts can arise in fixation, but they are clearly distinct from the manifestations of extrusion already described. Thus clear or granular vesicles of cytoplasm, resembling those seen in extrusion, have occasionally been seen arising in large numbers from the free border of the epithelium in preparations fixed in saline Flemming-without-acetic, or Champy. They are easily distinguished as fixation artefacts, for nothing like them was ever seen in life, and moreover they arose chiefly from the ciliated cells. In the few instances where they could be traced to gland cells, they had no obvious relation either to papillae or pore-rings. The fact that two fixatives which are noted for their faithful fixation of cytoplasm, can produce such effects, illustrates the necessity for correlating observations on fixed and living material (previously emphasized by Buchmann 1928), and for the maintenance of constant guard against artefacts.

The intimate co-operation between glands and ciliated cells in the accomplishment and control of extrusion is significant, as I have been unable to find such co-operation demonstrated elsewhere, though Polowzow (1903) supposed that the ciliated cells of the pharyngeal diverticulum of earthworms had a somewhat similar function. The co-operation is all the more interesting since the two types of cell commonly occur together in similar relationship in epithelia, and their co-operation in extrusion may perhaps be widespread. So far as the assistance given to extrusion by the ciliated cells is concerned, the process in earthworm parallels that described as extrusion by Needham (1897), occurring in certain cells in the gut of dragon-fly nymphs, and accomplished with the aid of compression resulting from the activities

of the surrounding cells. Again, it parallels instances where pressure assisting extrusion is said to be produced by the action of a specialized investment of muscle fibres, as in the sweat glands of ruminants (Brinkmann 1911), and the salivary glands of *Helix* (Pacaut & Vigier 1906), or by myo-epithelial cells (the so-called 'basket' or 'basal' cells) as in the salivary glands of mammals.

It is now appropriate to mention other suggestions that have been advanced as to the way in which extrusion occurs. Vignon (1899, 1901), Renaut (1911), Tchang Yung-Tai (1929), Fraser (1929) and many others, maintained that extrusion could occur without visible alteration in the gland. Others, such as Anderson (1884), Covell (1928) and Weatherford (1929), described extrusion taking place by cytoplasmic vacuoles in the secretory cells rising to the surface and bursting to discharge their contents into the lumen of the gland. Others again, such as Van Gehuchten (1890), Poyarkoff (1910), Brinkmann (1911), Hirsch (1918), Buchmann (1928), Jeffers (1935), Williams (1939) and De Robertis (1942), maintained that certain glands may extrude by more than one means. Such observations provide not only an indication that, as in *Lumbricus*, the process of extrusion in a particular type of gland may vary, but also a possible connecting link between what have seemed opposing views concerning extrusion Further, as in *Lumbricus*, the variation in behaviour of the sweat glands of ruminants and the gut glands of *Pyrausta* described by Brinkmann and Buchmann respectively is so marked as greatly to diminish the usefulness of the widely adopted division of glands into the 'holocrine' and 'merocrine' categories proposed by Ranvier (1887), even when used in conjunction with the more recently adopted intermediate category of 'apocrine' (see Maximov & Bloom 1942). Though the intestinal glands of *Lumbricus* usually behave as apocrine glands, only the tip being discharged during extrusion, this is by no means universal, for indications of holocrine behaviour occasionally appear in sections, where cells appear to be extruding their entire contents, and all grades between the two may be distinguished The only useful distinction that can be made between the glands is that given on p. 372, based on their rates of extrusion, but it is emphasized that even this depends on structural features outside the gland

The flexible behaviour of many glands, and more especially those reported by Jeffers (1935), Williams (1939), and De Robertis (1942), indicates the possibility of glands extruding in several distinct ways, dependent on physiological conditions. Thus, although the preceding account of extrusion in *Lumbricus* covers all the visible manifestations of the process yet observed, the possible existence of other means of discharge, which like the filtration or exudation processes envisaged by Vignon, Renaut, etc., would not necessarily be revealed by the technique used here, must always be borne in mind.

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EXPLANATION OF PLATE 18

(facing p. 362)

FIGURES 3 and 4. Photographs of portions of the typhlosolar epithelium of *Lumbricus* in section, showing the intracellular fibrils of the ciliated cells. Fixed Bonda, stained Heidenhain's iron-haematoxylin   *c.* constriction in gland cell owing to bulges in surrounding ciliated cells, *cil c i , cil c ii* ciliated cells, *d g c.* discharged gland cell; *i i f* insertion of intracellular fibril on basement membrane, *s cil.c i., s.cil c.ii.* localized swelling of ciliated cells producing constriction of gland cell. Other letters as in previous figures.

FIGURE 5. Photograph of a portion of the typhlosolar epithelium of *Lumbricus* seen in a section cut parallel to the free surface, to show the pore-rings and their relation to the intracellular fibrils which are seen in cross-section. Fixed in Goodrich's modification of Bouin's fluid, stained in Heidenhain's iron-haematoxylin. *A, A',* points where intracellular fibrils arch over to join the pore-ring. Other letters as in previous figures.

The buoyancy of plankton diatoms: a problem of cell physiology

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Observations on pure cultures of diatoms and the results of centrifuge experiments showed that under optimal conditions the vegetative cells have the same specific gravity as sea water.

The specific gravity of resting spores is significantly higher than that of sea water. Hence the specific gravity of the cell sap which fills the bulk of the vegetative cell and is expelled during formation of the resting spore is lower than that of sea water. The redu·ed weight (i e. weight under water) of resting spores of *Ditylum* was measured by means of the Cartesian diver balance. The mean value obtained was 0.00312γ per spore From calculations of the volume difference between the vegetative cell and the resting spore the specific gravity of the cell sap was estimated at 1 0202, i.e 0 0025 less than that of the sea water used

It is suggested that the mechanism underlying buoyancy in plankton diatoms is similar to that involved in the flotation of *Halicystis* and consists in the maintenance of very low concentrations of divalent ions in the cell sap, the result of a steady expenditure of energy

Introduction

Plant production in the sea is restricted to shallow inshore waters and to the upper water layers of the open sea where the intensity of submarine daylight is sufficient for the photosynthesis of organic material to exceed its consumption. For plankton diatoms in the English Channel compensation occurs during summer at a depth of about 45 m.; below that depth the diatoms consume more oxygen than they produce by photosynthesis (Jenkin 1937, Pettersson, Höglund & Landberg 1934). Since the centric plankton diatoms are incapable of active movement the question arises how they maintain themselves in the euphotic zone. This point has received little attention, and the answer given is usually based on Ostwald's general theory of plankton (Ostwald 1902). It has been taken for granted that the specific gravity of plankton diatoms is greater than that of sea water, as it is in most other plants and animals, but that their rate of sinking is reduced by frictional resistance due to their small size and the presence of flotation devices, such as thin long spines, and by the viscosity of the surrounding water. The structural adaptations, and possibly a reduced specific gravity due to the presence of oil, are believed to be sufficient to keep the diatoms in suspension long enough to enable them to grow and reproduce (Russell 1927; Sverdrup, Johnson & Fleming 1942). The following observations throw new light on the problem and necessitate a revision of the generally accepted view.

The specific gravity of vegetative cells

Plankton diatoms in pure cultures remain uniformly suspended throughout the water column and only sink to the bottom of the culture vessel when the population density becomes very high, or when the temperature rises to about 20° C, or when the culture becomes contaminated. This has been noted with cultures of *Chaetoceros pseudocrinitus, C. didymus, Coscinodiscus excentricus, Skeletonema costatum* and

Ditylum Brightwelli. Prolonged observations on individual diatoms in Petri dishes or, with the aid of a horizontal microscope, in rectangular glass cuvettes, showed that they either remain stationary, suspended in midwater, or drift slowly up and down with slight convection currents. *We may conclude, therefore, that under suitable physical conditions the plankton diatoms do not, in fact, sink, that their specific gravity is equal to that of sea water.* On the other hand, all students of marine biology are familiar with the fact that in rich plankton samples from tow nettings diatoms often form a thick sediment. They also tend to settle to the bottom in crowded cultures. Preserved diatoms sink in sea water with a velocity of 3 to 40 mm. per min. (Apstein 1910). Thus our conclusion that they have the same specific gravity as sea water only applies to living diatoms growing under favourable conditions, not to dead or physiologically damaged ones. One further qualification must be added, namely, that exceptionally narrow cells of *Ditylum* (diameter $20\,\mu$ or less), though capable of reproduction in cultures, seem to lose their buoyancy. Such narrow cells have not been observed in plankton samples.

THE SPECIFIC GRAVITY OF RESTING SPORES

Many neritic plankton diatoms survive unfavourable environmental conditions in the form of resting spores. As the vegetative cells disappear from the plankton of the upper water layers resting spores may be found in greater depths, sinking towards the bottom (Lohmann 1908, Gran 1912). The conditions for the formation of resting spores have been investigated in a few species, especially in *Ditylum* (Gross 1937, 1940a, 1940b; Bhatia 1940). As these studies are relevant to the problem of buoyancy, the results may be summarized here. When a resting spore is being formed the plasma membrane is retracted from the siliceous cell wall and the protoplast shrinks gradually to a small compact body, spherical in *Ditylum* though of different shape in some other species (figure 1). When a resting spore germinates an almost exactly reverse process takes place. Fine protoplasmic processes grow out to connect the spore with the shell. The spore elongates and expands while the processes become thicker until the protoplast regains its full turgidity and completely fills the old shell. This process of recovery lasts several hours. The resting spore consists of the more solid components of the cell, the cytoplasm, nucleus and chromatophores. The main change from the vegetative cell to the resting spore consists of the loss of cell sap which in *Ditylum* represents from 75 to 98 % of the cell volume, varying with the width of the cells. A resting spore which has dropped out of the shell or has been removed from it, expands in a similar way to form a spherical body of greatly increased volume, an 'auxospore', prior to the secretion of a new shell.

Ditylum undergoes resting-spore formation in crowded cultures at low light intensity and low temperature. Resting spores are, however, formed also as a result of very rapid plasmolysis in isotonic and hypotonic solutions of NaCl, $CaCl_2$ and sugars. In isotonic solutions of $NaCl + CaCl_2$ complete plasmolysis takes place in several hours instead of seconds, and a similar reaction occurs in unbuffered mixtures of NaCl, KCl, $MgCl_2$, $CaCl_2$ and $MgSO_4$, and also in sea water with

increased hydrogen-ion concentration. No plasmolysis takes place, however, in artificial sea water of pH 8, a value approximating to that of sea water. Cyanide and anaerobic conditions cause immediate plasmolysis, and resting spores are completed in several hours. In all these experiments full recovery was obtained on transferring the resting spores to clean sea water, irrespective of light conditions. Vegetative cells plasmolyse slowly in darkness: the retraction of the cell membrane begins after 15 to 22 hr. and resting spores are completed in 7 to 12 days (Gross 1940a). Very similar effects are produced by suitable concentrations of urethane (Bhatia 1940) which are known to inhibit assimilation in algal cells (War-

<center>(a)   (b)</center>
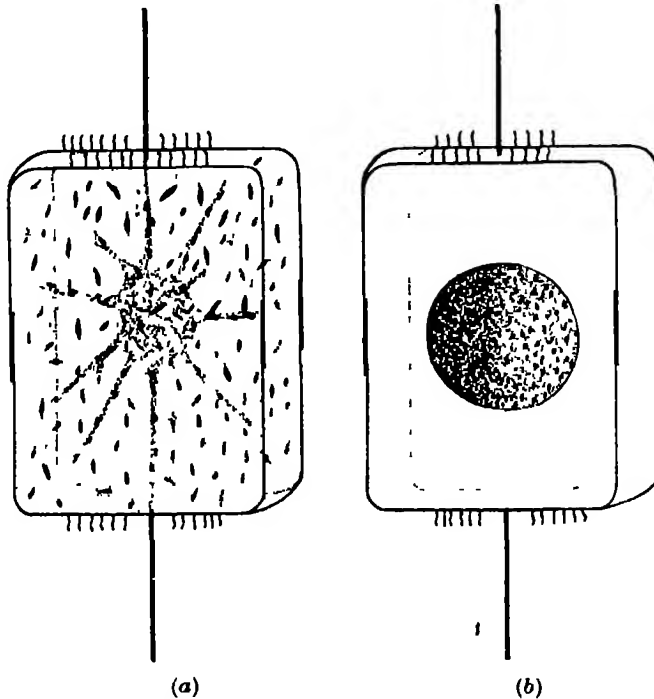
FIGURE 1. Diagram of *Ditylum Brightwelli* (a) vegetative cell, (b) resting spore.

burg 1919). Resting spores formed under the influence of urethane or lack of light recover only when exposed to light. These observations lead to the conclusion that, apart from the presence of NaCl, $CaCl_2$ and a hydrogen-ion concentration of about pH 8, some metabolic activity is required to maintain the vegetative cells in their expanded, turgid state, an activity which is inhibited by the exhaustion of oxidizable material (in darkness and in urethane) or by the inhibition of cell respiration (in cyanide and de-oxygenated sea water).

To return now to the problem of buoyancy, we find that resting spores invariably sink while vegetative cells under identical conditions remain suspended. *Resting spores (with and without their shells) have, therefore, a specific gravity significantly greater than that of sea water.* In sea water of a specific gravity of 1·0223 and at a temperature of 14° C, *Ditylum* resting spores sink at a rate of 4 to 6 mm. per min., and would therefore, in the absence of currents, sink about 7 m. in 24 hr. When

centrifuged at the very moderate speed of a hand centrifuge they accumulate at the bottom of the tube in less than 2 min., while vegetative cells of both *Ditylum* and *Chaetoceros*, taken from young, vigorously growing cultures, may be centrifuged at 1500 rotations per min. for 10 min. or more without more than a fraction of the population being deposited in the bottom of the tube. In the sea, resting spores are never present in the plankton for any length of time comparable to the periods during which vegetative cells populate the upper-water layers. This confirms the observation that resting spores have a specific gravity higher than sea water, and sink in it in spite of possessing the same structural flotation devices as the vegetative cells.

From all these observations we may conclude that *the cell sap which fills the bulk of the vegetative cell and is expelled during the formation of the resting spore has a specific gravity less than that of sea water.* Short of an analysis of the cell fluid, which would be difficult, some idea of the nature and properties of the cell sap might be deduced from measurements of the specific gravity of resting spores. Experiments in which spores of *Ditylum* were centrifuged in solutions of gum in sea water of different specific gravity proved unsatisfactory, the results suggesting an absurdly high value. However, the recent development of the Cartesian diver balance by Zeuthen (1947) has presented a suitable method for the determination of the weight in water of small objects.

THE REDUCED WEIGHT OF RESTING SPORES AND THE SPECIFIC GRAVITY OF THE CELL SAP

The diver balance is a modification of the Cartesian diver respirometer developed by Linderstrøm-Lang and his collaborators. It consists of a scale, 1 to 2 mm. in diameter, blown from polysterine and mounted on a minute glass chamber which is drawn out into a tail-like narrow capillary. An air bubble with some water is enclosed in the chamber. The diver is suspended in a flotation vessel which is connected to a manometer. Pressure variations in the flotation vessel are transmitted to the air bubble through the capillary. The change in equilibrium pressure due to charging the balance with an unknown weight is compared with that resulting from charging it with small polysterine beads of known reduced weight.

The diver balance weighs the reduced weight (R.W.) of small stationary organisms, i.e. their surplus weight when suspended in their normal medium.

Ditylum used for the weighings were taken from a culture of uniformly broad, rectangular cells of square base. Variations in size consisted almost entirely of differences in length according to the age of the cells. Their regular shape (figure 1) facilitates accurate measurements. Volumes were calculated from measurements of length and width of cells and from the diameter of resting spores. The relevant data obtained from 100 cells and resting spores are given in table 1.

Resting spores were prepared by treating the vegetative cells with isotonic sodium chloride and subsequently washing them repeatedly in order to remove the mucus (possibly of bacterial origin) adhering to the shells of the spores. They were then transferred to the culture medium and finally, with a drop of medium, on to the scale of the diver balance.

TABLE 1. MEAN SIZES OF 100 VEGETATIVE CELLS AND 100 RESTING SPORES OF *DITYLUM*

	length in μ	width in μ	volume (lw^2) in 1000 μ^3
vegetative cells	$127 \cdot 6 \pm 4 \cdot 0$	$102 \cdot 7 \pm 0 \cdot 8$	1360 ± 50
	diameter (d) in μ		volume ($\frac{4}{3}\pi(\frac{1}{2}d)^3$) in 1000 μ^3
resting spore	$58 \cdot 5 \pm 0 \cdot 57$		110 ± 4

In three experiments performed on 120, 240 and 394 spores the average R.W. of a single spore was found to be 0·00280, 0·00342 and 0·00314γ respectively (mean 0·00312γ). The volume of cell sap which is expelled from the cell when the resting spore is formed equals the difference between the volume of the cell and that of the resting spore, i.e. $1 \cdot 25 \times 10^{-3} \mu l$. Evidently in the vegetative cell this volume of cell sap is light enough to 'carry' the whole diatom in the sea. Since thus $1 \cdot 25 \times 10^{-3} \mu l$. carry 0·00312γ, 1μl. will carry 2·5γ R.W., which therefore is the difference in weight of 1μl. sea water and 1μl. cell sap. At 22° C (the temperature of two of our experiments) the specific gravity of the sea water (salinity 33·1 °/oo) used was 1·0227; hence the specific gravity of the cell sap was 1·0227 − 0·0025 = 1·0202.

From the results of studies on *Valonia* and *Halicystis* by Osterhout and his co-workers, which will be discussed below, it seemed possible that the reduction in specific gravity of the diatom cell sap may be brought about by the elimination or radical reduction in the amount of divalent ions. Therefore, solutions of the main constituents of sea water were prepared in different combinations and their specific gravity determined (see table 2). They were made up in the same proportions in which they occur in sea water (Harvey 1945). Solution V represents 'simplified artificial sea water' (32·5°/oo). Salts present in the sea in very small amounts were not considered. The solutions were adjusted to isotonicity by Baldes's (1934) method for determining total osmotic pressure (accuracy about 1%).

TABLE 2. SPECIFIC GRAVITY OF SALT SOLUTIONS ISOTONIC WITH 32·5°/oo SEA WATER

solution	salts present	specific gravity at 22° C
I	NaCl	1·0199
II	NaCl, KCl	1·0201
III	NaCl, KCl, CaCl$_2$	1·0211
IV	NaCl, KCl, CaCl$_2$, MgCl$_2$	1·0221
V	NaCl, KCl, CaCl$_2$, MgCl$_2$, Na$_2$SO$_4$	1·0235

When the divalent ions present in sea water are added to a solution of NaCl + KCl there is a marked increase in the specific gravity of the solution. Addition of CaCl$_2$, MgCl$_2$ and Na$_2$SO$_4$ bring about an increase in specific gravity by 0·0034, that of MgCl$_2$ and Na$_2$SO$_4$ an increase by 0·0024. Na$_2$SO$_4$ alone accounts for an increase in specific gravity of 0·0014 We have seen above that the specific gravity of the cell sap of *Ditylum* is probably 0·0025 lower than that of the sea water bathing the cell. It would seem, therefore, that the buoyancy of *Ditylum* may be accounted for by a reduction of divalent ions in the cell sap to very low concentrations comparable

to those found in *Halicystis* and *Valonia* (Osterhout & Dorcas 1925). This hypothesis involves the assumption, however, that, as in *Halicystis*, K^+ is not accumulated in the sap, KCl solutions being heavier than isotonic NaCl solutions. Thus a KCl solution isotonic with solution I of table 2 has a specific gravity of 1·0233.

DISCUSSION

Among the studies of flotation phenomena in other organisms those concerned with the cystoflagellate *Noctiluca* and the large multinuclear marine algae *Valonia* and *Halicystis* are the most important in their relevance to the problem of buoyancy of plankton diatoms.

Noctiluca is found floating on the surface of the sea. E. B. Harvey (1917) suggested that its lower specific gravity is due to the hypotonicity of the cell sap. Krogh (1939), however, reviewing the literature, thinks it more likely that the cell sap is isotonic with sea water, but that it is light, due to a different ionic composition of its cell sap. In his opinion *Noctiluca* floats owing to an accumulation of the light ion NH_4^+, retained in the cell sap because of a low pH (pH 3 according to Gross 1934). Krogh lays much stress upon the work of Goethard & Heinsius (1892) who have demonstrated the presence of NH_4^+ in the cell sap by qualitative tests. It may be more significant, however, that their tests for sulphate were negative. The absence of sulphate alone would make the cell sap lighter than sea water. As suggested by Krogh experiments on *Noctiluca* should be repeated with modern methods.

According to Osterhout & Dorcas (1925) *Valonia* and *Halicystis* differ in that the former sinks while the latter floats in sea water. The abundant cell sap of both these algae contains extremely little organic matter and is almost isotonic with sea water, but differs from it in ionic composition. In the cell sap of both organisms SO_4^- is present in traces only. The same is true for Mg^{++} in *Valonia*, in *Halicystis* Mg^{++} is present in low concentrations only. Ca^{++} is present in both cell saps but again in lower concentrations than in sea water (table 3). The general tendency is to keep divalent ions out of the cell sap. K^+ is accumulated in *Valonia* but not in *Halicystis*. The pH of the cell sap is 5·1 (*Halicystis*) and 5·9 (*Valonia*). NH_4^+ is present in extremely small amounts.

TABLE 3 (from Osterhout, 1933). MOLECULAR COMPOSITION IN PERCENTAGE OF Cl

	sea water	sap of *Valonia macrophysa*	sap of *Halicystis Osterhouti*
Cl	100	100	100
Na	85·87	15·08	92·4
K	2·15	86·24	1·01
Ca	2·05	0·285	1·33
Mg	9·74	trace	2·77
SO₄	6·26	trace ?	trace

It has been mentioned before that the specific gravity decreases when the concentration of divalent ions is reduced. When the concentration of K^+ is increased at the expense of Na^+ the specific gravity again increases. While therefore the specific gravity of sea water was found to be 1·0277, the cell sap of *Halicystis* was

lighter, viz. 1·0250, and the cell sap of *Valonia* heavier, viz. 1·0290. Salt solutions made up in accordance with the analyses gave values of specific gravity of 1·0285 for artificial sea water, of 1·0252 for the artificial sap of *Halicystis*, and 1·0285 for that of *Valonia*. Osterhout & Dorcas (1925) concluded therefore that the difference in buoyancy of the two organisms was due to the differences in ionic composition of their cell saps. Whereas they ascribe the flotation of *Halicystis* solely to the lack of sulphate ion, our data suggest that the concentration of other divalent ions may also be reduced in the cell sap of *Ditylum*.

It may be mentioned that if *Valonia* is placed in sea water containing low concentrations of NH_4Cl (1 mM), NH_4^+ exchanges with K^+ in the cell sap. After a while the inside concentration of NH_4^+ is many times higher than the outside concentration and the cells float because NH_4^+ is light. These floating cells continue to grow indefinitely (Cooper, Dorcas & Osterhout 1929). We must remember, however, that normally the sap of *Valonia* and *Halicystis* is free of NH_4^+, so that the normal flotation in *Halicystis* cannot be due to an accumulation of this ion.

Noctiluca appears to be in osmotic equilibrium with the outside medium Its volume increases when transferred into dilute sea water (Harvey 1917), whereas *Ditylum* plasmolyses in the same medium and even in a fresh-water medium. On the other hand, the similarity in behaviour of *Ditylum* and *Halicystis* is so striking that one is tempted to assume a similarity in the mechanisms of flotation in the two systematically widely separated organisms, viz. the exclusion of most of the divalent ions from the cell sap. These considerations do not exclude other possible explanations. If, however, in *Ditylum* light ions such as H^+ or NH_4^+ were to accumulate in sufficient amounts to 'carry' the cell, high concentrations (of the order of 0·1 N) would be required Such high concentrations are improbable.

An ionic composition different from that of the surrounding medium can be maintained only by osmotic work, which will require a constant supply of energy. As regards *Halicystis*, no experiments seem to have been made to show whether expenditure of energy is involved in its flotation Cyanide has no effect on the flotation ability of *Noctiluca* (Goethard & Heinsius 1892). The effects of darkness, of cyanide and of lack of oxygen on *Ditylum*, on the other hand, indicate that energy derived from continuous oxidation processes is required for the maintenance of its flotation mechanism.

Whatever may be its composition in *Ditylum* we must regard the maintenance of cell sap of specific gravity lower than sea water as the critical factor in its buoyancy and presumably also in the buoyancy of other plankton diatoms. Other factors, such as structural flotation devices, could no more maintain them in the euphotic zone than they do the resting spores. Physical factors, particularly the viscosity of the water, are doubtless of importance, it has been mentioned already that buoyancy is reduced at higher temperatures and consequent lower viscosity. Further studies of both the physiological and physical factors involved are likely to throw light on some important ecological problems, such as the succession of different diatom species in the plankton or the disappearance of diatoms from the upper water layers while much phosphate still remains in these layers (Marshall & Orr 1927).

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The passage of antibodies from the maternal circulation into the embryo in rabbits

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Active immunity to *Brucella abortus* was induced in adult female rabbits. They were mated a week after the last injection of antigen and were killed and the yolk-sac contents of the embryos tested for agglutinins 8¼ days after copulation. Specific agglutinins were found to be present in the yolk-sac contents in all cases. The titre varied significantly from embryo to embryo in the same litter, and was in some as high as that in the maternal serum at the time of killing.

Passive immunity to *Br. abortus* was imparted to female rabbits 7 to 9 days pregnant by intravenous injection of immune serum of high titre. The rabbits were killed and the yolk-sac fluid of the embryos tested for agglutinins 10 to 17 hr after injection. Specific agglutinins were present in most of the embryos from five of the six rabbits injected before 8 days post-coitum. All the embryos in the sixth rabbit were regressing. Specific agglutinins were not found in any of the embryos from two rabbits injected after 9 days post-coitum, by which time the yolk-sac fluid has ceased to increase in volume. Positive results were obtained both when rabbit and bovine immune sera were used.

Active immunity to *Br. abortus* was induced in pregnant rabbits by injections beginning after the 15th day post-coitum. The serum of the newborn young, removed from their immune mothers before they had suckled, was tested and specific agglutinins were found to be present with a titre corresponding to that of the maternal serum.

It was concluded that agglutinins, whether actively or passively acquired, pass freely from the maternal circulation into the yolk-sacs of 7- and 8-day rabbit embryos. This constitutes a delicate test of the passage of protein without alteration through the yolk-sac wall. The yolk-sac wall does not appear to be selective, since it is at least as permeable to foreign proteins as it is to those of maternal origin. Agglutinins pass from the maternal circulation into the embryo after the disappearance of the bilaminar wall of the yolk-sac also, either by way of the yolk-sac splanchnopleur or the allantochorionic placenta or both. The bearing of these results on current theories of placental permeability are discussed.

1. Introduction

It has been shown (Brambell & Hemmings 1948) by means of electrophoretic and ultracentrifuge studies, that the yolk-sac fluid of rabbit embryos 7 and 8 days post-coitum contains albumen, α-, β- and γ-globulins and fibrinogen in similar proportions relative to each other as in the maternal plasma. The known immunological significance of γ-globulins in the conveyance of specific antibodies suggested that proof of the maternal origin of these globulins could be obtained if one or other such specific antibody could be identified in yolk-sac fluid. The simplicity with which the agglutinin can be detected made it the antibody of choice for this work, and since *Brucella abortus*, the causal organism of a specific 'infectious abortion' in cattle, is capable of stimulating antibody production of this type in rabbits, this was used as the necessary antigen. The result of the initial experiments, undertaken with this object, led to further investigations. The work described herein is the outcome.

2. TECHNIQUE

The methods employed of killing and collecting the yolk-sac fluid have been described already (Brambell & Hemmings 1948). A standardized carbolized suspension of *Br. abortus*, as used in routine agglutination tests of bovine sera, was employed, both for inducing active immunity in the rabbits and for setting up the agglutination tests. Active immunity was induced by three successive subcutaneous inoculations with 0·5, 0·8 and 1·0 ml. of the antigen at intervals of 3 days between each dose. Blood samples were taken from the ear vein and were tested for specific circulating antibody before the first and after the last inoculation. Passive immunity was obtained by the injection of bovine sera of known titre (1/640 to 1/1280) which were available in the laboratory and one of high titre (1/5120) which was specially prepared for us by Professor Dalling. Subcutaneous injection of 10 ml. failing to produce a sufficient titre in the circulation owing to the rate of absorption relative to the rate of disappearance being too low, intravenous injection was employed. It was found that 5 to 6 ml. was the maximum dose that could be administered with safety by this route, as 10 ml. resulted in death in 2 min.

The standardized agglutination test as used in the detection of *Br. abortus* agglutinins in bovine sera was used. Serial dilutions of 1/10 to 1/5120 of the test fluid were set up and equal volumes of the antigen added. The tubes were then incubated at 37° C for 20 hr. and read against a light of standard intensity. Readings were recorded as follows:

+ + +   Complete agglutination. Heavy sedimentation of organisms. Supernatant fluid clear.

  + +   Partial or incomplete agglutination. Incomplete sedimentation of organisms. Supernatant fluid slightly hazy.

    +   Slight flocculation but little sedimentation of organisms. Some clearing of supernatant fluid.

    —   No change. Fluid maintaining original opacity.

3. OBSERVATIONS

Passage of homologous antibody into the yolk-sac fluid

Two experiments were performed. Four rabbits, one control (13,766) and three experimental (13,716, 13,719 and 13,722), were employed in the first experiment. Active immunity was induced in the experimental animals which were mated on the 8th day after the last injection of antigen, the control being mated at the same time They were killed at 8 days 15 hr. post-coitum by a lethal dose of Nembutal administered intravenously. All four were pregnant and the yolk-sac fluid of one embryo only from each animal was tested.

Three experimental rabbits (13,764, 13,765 and 13,807) were employed in the second experiment. Active immunity was induced and they were mated on the 6th day after the last injection of antigen. They were killed at 8 days 17 hr. post-coitum by bleeding, under ether anaesthesia following intravenous injection of Nembutal. All three were pregnant and the yolk-sac fluid from each embryo was tested.

25-3

TABLE 1. THE PASSAGE OF HOMOLOGOUS AGGLUTININS FROM THE MATERNAL CIRCULATION INTO THE YOLK-SAC FLUID

ref. number	test sample	titre								
		1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560
13,766 (control)	maternal serum: before immunization	-	-	-	-	-	-	-	-	-
	1 day before mating	-	-	-	-	-	-	-	-	-
	at killing	-	-	-	-	-	-	-	-	-
	yolk-sac fluid: embryo 1	-	-	-	-	-	-	-	-	-
13,719	maternal serum: before immunization	-	-	-	-	-	-	-	-	-
	1 day before mating	+	+	+	+	+	+	+	-	-
	at killing	+	+	+	+	+	+	+	-	-
	yolk-sac fluid: embryo 1	?	+	+	-	-	-	-	-	-
13,716	maternal serum: before immunization	-	-	-	-	-	-	-	-	-
	1 day before mating	+	+	+	+	+	+	+	-	-
	at killing	+	+	+	+	+	+	+	+	+
	yolk-sac fluid: embryo 1	?	+	+	+	+	+	+	+	+
13,722	maternal serum: before immunization	-	-	-	-	-	-	-	-	-
	1 day before mating	+	+	+	+	+	+	+	-	-
	at killing	+	+	+	+	+	+	+	+	+
	yolk-sac fluid: embryo 1	+	+	+	+	+	+	+	-	-

? = partial clotting of yolk-sac fluid rendering interpretation difficult.

TABLE 2. THE PASSAGE OF HOMOLOGOUS AGGLUTININS FROM THE MATERNAL CIRCULATION INTO THE YOLK-SAC FLUID

ref. number	test sample	titre								
		1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560
13,764	maternal serum: before immunization	−	−	−	−	−	−	−	−	−
	3 days before mating	+	+	+	+	+	+	+	+	+
	at killing	+	+	+	+	+	+	+	+	+
	yolk-sac fluid: embryo 1R	+	+	+	+	+	+	+	−	−
	embryo 2R	+	+	+	+	+	+	+	−	−
	embryo 1L	+	+	+	+	+	+	−	−	−
	embryo 2L	+	+	+	+	+	+	+	+	−
	embryo 3L	+	+	+	+	+	+	+	−	−
	embryo 5L	+	+	+	+	+	+	+	−	−
	embryo 6L	+	+	+	+	+	+	+	−	−
13,765	maternal serum: before immunization	−	−	−	−	−	−	−	−	−
	3 days before mating	+	+	+	+	+	+	+	+	−
	at killing	+	+	+	+	+	+	+	+	−
	yolk-sac fluid: embryo 1R	+	+	+	+	+	+	−	−	−
	embryo 2R	?	+	+	+	+	+	−	−	−
	embryo 3R	?	+	+	+	+	+	+	−	−
	embryo 4R	?	+	+	+	+	+	−	−	−
	embryo 5R	+	+	+	+	+	+	+	−	−
	embryo 6R	+	+	+	+	+	+	−	−	−
	embryo 7R	+	+	+	+	−	+	−	−	−
	embryo 1L	+	+	+	+	+	+	−	−	−
13,807	maternal serum: before immunization	−	−	−	−	−	−	−	−	−
	3 days before mating	+	+	+	+	+	+	+	+	+
	at killing	+	+	+	+	+	+	+	+	+
	yolk-sac fluid: embryo 1R	+	+	+	+	+	+	+	+	−
	embryo 3R	+	+	+	+	+	+	+	+	+
	embryo 1L	+	+	+	+	+	+	+	+	−
	embryo 3L	+	+	+	+	+	+	+	+	+
	embryo 4L	+	+	+	+	+	+	+	+	+
	embryo 5L	+	+	+	+	+	+	+	+	+

? = partial clotting of yolk-sac fluid rendering interpretation difficult.

The results of the tests of the maternal sera and of the yolk-sac fluids are given in tables 1 and 2 (see pp. 392, 393). Specific agglutinins were found to be present in the yolk-sac fluid of all the embryos tested from all six experimental animals and were absent from the control. Attention is drawn to what is regarded as a surprisingly high titre in the yolk-sac fluid in relation to that in the maternal serum at the time of killing and to the clearly significant difference in titre between the yolk-sac fluids of the various embryos of a litter, which can be seen in each litter in table 2.

Passage of heterologous antibody into the yolk-sac fluid

The observation that homologous antibody passes freely into the yolk-sac through the trophoblast and the entoderm, presumably, of the bilaminar omphalopleur suggested that it was desirable to test whether heterologous antibody behaved in a similar manner. Bovine serum having a high titre of agglutinin for *Br. abortus* was injected intravenously into 7- to 9-day pregnant rabbits, the blood of which was previously tested and found to be free of agglutinins. The blood was again tested and the animals were killed 10 to 17 hr. after injection by the magnesium chloride method, and the yolk-sac fluid of the embryos was collected and tested. Four such experiments were performed.

The first experiment included two animals, one (13,844) being used as a control and injected with 5 ml. immune rabbit serum of 1/1280 titre, and the other (13,802) injected with 5 ml. immune bovine serum of equivalent titre at 7 days 12 hr. post-coitum, so as to obtain approximately equal titres in the maternal blood. Both were killed at 8 days 1 hr. post-coitum and the results are given in table 3.

The second experiment included two animals (13,803 and 13,808), both of which were injected with 5 ml. of bovine serum of 1/1280 titre at 7 days 14 hr. post-coitum. They were killed at 8 days 1 hr. and 8 days 0 hr. post-coitum respectively. The results are given in table 4. All the embryos in the litter of 13,808 were abnormally small, and difficulty was experienced in collecting fluid from them. From some no fluid was obtained and from others so little that it was necessary to resort to combining fluid from two or more embryos to obtain sufficient for testing. Only one of the three samples showed any trace of agglutination, and even this could not be regarded as significant. It is probable that all the embryos in this litter were reabsorbing, and that death had occurred at or before the time of injection.

The third experiment included two animals (13,778 and 13,781), both of which were injected with 5 ml. of bovine serum of 1/640 titre at 9 days 1 hr. post-coitum. They were killed at 9 days 13 hr. and 9 days 12 hr. post-coitum respectively. The results are given in table 5. Although all the embryos tested appeared normal and perfectly healthy, none, from either litter, gave any positive reaction at the lowest titre of 1/10. This result is in accord with those obtained with Evans blue (Brambell & Hemmings 1948), in which the concentration of dye attained in the yolk-sac fluid declines with age at injection and is not detectable when injected at or after 8 days 20 hr.

The fourth experiment included two animals (13,771 and 13,772), both of which were injected with 5 ml. of bovine serum of 1/5120 titre at 7 days 22 hr. post-coitum. They were killed at 8 days 15 hr. and 8 days 13 hr. post-coitum respectively. The results are given in table 6.

TABLE 3. THE PASSAGE OF HETEROLOGOUS AGGLUTININS FROM THE MATERNAL CIRCULATION INTO THE YOLK-SAC FLUID

ref. number	test sample	titre					
		1/10	1/20	1/40	1/80	1/160	1/320
13,844 (control)	maternal serum: before injection	−	−	−	−	−	−
	after injection	+++	+++	+++	−	−	−
	at killing	++	+	+	−	−	−
	yolk-sac fluid. embryo 1R	+++	+	−	−	−	−
	embryo 3R	+++	−	−	−	−	−
	embryo 1L	+++	++	−	−	−	−
	embryo 2L	+	+	−	−	−	−
	embryo 3L	+++	+	−	−	−	−
	embryo 4L	−	−	−	−	−	−
	embryo 5L	−	−	−	−	−	−
13,802	maternal serum: before injection	−	−	−	−	−	−
	after injection	+++	+++	++	+	+	−
	at killing	+++	+++	++	+	−	−
	yolk-sac fluid. embryo 1R	+++	+	++	−	−	−
	embryo 2R	+++	−	−	−	−	−
	embryo 3R	+++	+++	−	−	−	−
	embryo 4R	+++	+++	+	−	−	−
	embryo 5R	++	−	++	+	−	−
	embryo 7R	+++	+++	+	−	−	−
	embryo 1L	+++	+++	−	−	−	−

TABLE 4. THE PASSAGE OF HETEROLOGOUS AGGLUTININS FROM THE MATERNAL CIRCULATION INTO THE YOLK-SAC FLUID

ref. number	test sample	titre					
		1/10	1/20	1/40	1/80	1/160	1/320
13,803	maternal serum: before injection	–	–	–	–		–
	after injection	+	+	+++	++	+	–
	at killing	+	+	++	+	–	–
	yolk-sac fluid: embryo 1R	+	+	+++	–	–	–
	embryo 2R	+	+	+++	–	–	–
	embryo 3R	+	+	+++	–	–	–
	embryo 4R	+	+	+++	–	–	–
	embryo 1L	+	++	+	–	–	–
	embryo 2L	+	+	++	–	–	–
	embryo 3L	+	+	++	–	–	–
	embryo 4L	+	+	+	–	–	–
13,808	maternal serum: before injection	–	–	–	–	–	–
	after injection	+	+	++	++	+	–
	at killing	+	+	++	++	–	–
	yolk-sac fluid: embryo 2R	–	–	–	–	–	–
	embryos 1R, 3R, 1L	–	–	–	–	–	–
	embryos 3L, 4L	+	–	–	–	–	–

Passage of homologous antibody into the embryo after the 15th day of gestation

The demonstration of the presence of antibodies in the yolk-sac fluid raised the question of whether the yolk-sac contained the whole supply to the embryo. If so, then no more maternal antibodies should reach the embryo after the 15th day of gestation, by which time the bilaminar omphalopleur in the rabbit has disappeared and the entoderm of the yolk-sac splanchnopleur is exposed to the uterine lumen. Any subsequent absorption of maternal antibodies might take place either through the yolk-sac splanchnopleur, the entoderm of which is in contact with, but not fused to, the reformed uterine epithelium or through the allantochorionic placenta. Rodolfo (1934) had shown that the titre of *Br. abortus* agglutinin and of haemolysin to sheep red blood corpuscles in the blood of foetuses of immunized rabbits rises continuously from the 22nd day of gestation to full term, the rate of increase reaching a maximum on the 27th day. Since in his experiments the rabbits were immunized before mating his results appeared to require confirmation on the question at issue. Consequently it was decided to induce active immunity to *Br. abortus* in pregnant rabbits after the 15th day of gestation and to test the blood of the newborn young before they had suckled. Five rabbits were employed, two (13,740 and 13,769) being used as controls and three (13,739, 13,760 and 13,768) being immunized. The three experimental animals were given the first of three doses of antigen at 14 days 16 hr. post-coitum and the last at 27 days. The animals were kept under observation while littering, and the young to be tested at birth were removed as soon as they were born before they had suckled. A newborn young from each of two of the immunized animals was fostered on a control and tested 2 days later. Blood to be tested was removed by cardiac puncture under ether anaesthesia. The results are given in table 7.

4. DISCUSSION

The experimental results demonstrate that agglutinin for *Br. abortus*, whether actively or passively acquired, passes freely from the maternal circulation into the yolk-sac cavities of embryo rabbits on the 7th and 8th days post-coitum. The fact that the agglutinin passively acquired by the mother, inoculated with immune serum, is to be found a few hours later in the yolk-sac fluid of the embryos precludes all possibility that it was formed there. The retention of its immunological identity by the agglutinin after its passage through the yolk-sac wall, presumably the bilaminar omphalopleur, constitutes the most delicate test that we could devise to prove that protein remains unaltered in transit. This evidence, in addition to that presented already (Brambell & Hemmings, and McCarthy & Kekwick 1948) regarding the other plasma proteins, can leave little room for doubt that proteins can pass unchanged and rapidly through the yolk-sac wall of the embryo.

It was thought possible that the embryonic trophoblast might display specificity in the selection of proteins, and that it might be permeable only to homologous proteins. The experiments with bovine agglutinating sera negative this supposition and show that the yolk-sac wall is at least as permeable to the heterologous, as it is to the homologous, protein.

TABLE 5. THE PASSAGE OF HETEROLOGOUS AGGLUTININS FROM THE MATERNAL CIRCULATION INTO THE YOLK-SAC FLUID

ref. number	test sample	titre					
		1/10	1/20	1/40	1/80	1/160	1/320
13,778	maternal serum: before injection	—	—	—	—	—	—
	after injection	+ + +	+ + +	+ + +	+	—	—
	at killing	+ + +	+ +	—	—	—	—
	yolk-sac fluid: each of 7 embryos	—	—	—	—	—	—
13,781	maternal serum: before injection	—	—	—	—	—	—
	after injection	+ + +	+ + +	+ + +	+ +	—	—
	at killing	+ +	+ +	+ +	—	—	—
	yolk-sac fluid: each of 9 embryos	—	—	—	—	—	—

TABLE 6. THE PASSAGE OF HETEROLOGOUS AGGLUTININS FROM THE MATERNAL CIRCULATION INTO THE YOLK-SAC FLUID

ref. number	test sample	titre							
		1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280
13,771	maternal serum: before injection	—	—	—	—	—	—	—	—
	after injection	+ + +	+ + +	+ + +	+ + +	+ + +	+	—	—
	at killing	+ + +	+ + +	+ + +	+ + +	+ +	—	—	—
	yolk-sac fluid: embryo 2L	+ + +	+ + +	+ + +	+	—	—	—	—
	embryo 3L	+ + +	+ + +	+ + +	+	—	—	—	—
	embryo 4L	+ + +	+ + +	+ +	+	—	—	—	—
	embryo 5L	+ + +	+ + +	+ + +	+ + +	—	—	—	—
13,772	maternal serum: before injection	—	—	—	—	—	—	—	—
	after injection	+ + +	+ + +	+ + +	+ + +	+ + +	+ +	—	—
	at killing	+ + +	+ + +	+ + +	+ + +	+ +	+	—	—
	yolk-sac fluid: embryo 1R	+ + +	+ + +	+ + +	+ +	+	—	—	—
	embryo 2R	+ +	+ +	+ +	+	—	—	—	—
	embryo 3R	+	+	+	—	—	—	—	—
	embryo 4R	+ +	+ +	+ +	—	—	—	—	—
	embryo 1L	+ +	+ +	+ +	—	—	—	—	—
	embryo 2L	+ +	+ +	+ +	+	—	—	—	—
	embryo 3L	+ +	+ +	+ +	+	—	—	—	—

TABLE 7. THE PASSAGE OF HOMOLOGOUS AGGLUTININS FROM THE MATERNAL CIRCULATION INTO THE EMBRYO AFTER THE 15TH DAY OF GESTATION

ref. number	test fluid	titre								
		1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560
13,740 (control)	maternal serum: before mating	—	—	—	—	—	—	—	—	—
	27th day of gestation	—	—	—	—	—	—	—	—	—
13,769 (control)	maternal serum: before mating	—	—	—	—	—	—	—	—	—
	27th day of gestation	—	—	—	—	—	—	—	—	—
13,739	maternal serum: before mating	—	—	—	—	—	—	—	—	—
	27th day of gestation	+++	+++	+++	+++	+++	+++	+++	++	+
	serum of newborn young	+++	+++	+++	+++	+++	+++	+++	+	—
	serum of 2 days 18 hr. young suckled by 13,769	+++	+++	+++	+++	+++	+++	+++	—	—
13,760	maternal serum. before mating	—	—	—	—	—	—	—	—	—
	27th day of gestation	+++	+++	+++	+++	+++	+++	—	—	—
	serum of newborn young	+++	+++	+++	++	++	+	—	—	—
	serum of 2 days 15 hr. young suckled by 13,740	+++	+++	+++	+++	+++	+++	+++	—	—
13,768	maternal serum: before mating	—	—	—	—	—	—	—	—	—
	27th day of gestation	+++	+++	+++	+++	+++	+++	+++	+++	+
	serum of newborn young	+++	+++	+++	+++	+++	++	++	—	—

The blastocyst of the rabbit is expanding rapidly on the 7th and 8th days. This expansion is due, almost entirely, to augmentation of the yolk-sac fluid, since the volume of embryonic tissue is only a small fraction of that of the yolk-sac contents. The membrane forming the thin wall of the yolk-sac possesses the remarkable property of being freely permeable to all the plasma proteins, which pass through it apparently unaltered and in proportions which appear to be relative to their concentrations and not to their molecular size, as the blastocyst is expanding.

The results of many of the experiments display variations in titre of agglutinins in the yolk-sac fluid among the several embryos in a litter, which are clearly significant. Should this variation mean that some of the blastocysts admit globulins more freely than others during their development, then this finding might well have additional importance in studies of intra-uterine immunity.

A great deal of work has been done on the transmission of antibodies from mother to young in mammals, and since the extensive literature of the subject has been reviewed by several authors (Needham, 1931, Marrack 1946; McGirr 1947), to whom reference may be made, a brief summary of the present status of the problem will suffice here. Young mammals appear to derive, initially, all their antibodies from the mother. According to present knowledge, limited to a comparatively few species, they fall into two groups, depending on whether they obtain all their maternal antibodies after birth, through the colostrum, or whether they obtain them mainly before birth, through the placenta.

Cattle, sheep, goats, horses and pigs belong to the first group (Mason, Dalling & Gordon 1930; Marrack 1946; McGirr 1947). The newborn young of these species have no antibodies in their blood before they have suckled. The mother secretes antibodies in the colostrum, the titre of which is as high, or higher than, that of the maternal serum. This is true both of homologous antibodies, actively formed in the body of the mother, and of heterologous antibodies injected into her body shortly before parturition. The antibodies, whether homologous or heterologous, pass rapidly from the gut into the circulation of the newborn young. So rapid is the passage that the titre in the serum of the newborn may equal, or even exceed, that of the mother a few hours after the first feed. Thereafter this permeability of the gut of the newborn young to antibodies rapidly declines and is soon lost.

Man, rabbits and guinea-pigs belong to the second group, the young deriving the greater part of their antibodies from the mother while still in the uterus, though these may be augmented by secretion in the colostrum and absorption through the gut after birth. Both homologous and heterologous antibodies can pass into the embryos in these animals.

The view was advanced by Kuttner & Ratner (1923) that the permeability of the placenta is related to its structure. Grosser's (1927) well-known classification of allantochorionic placentae is based on the number of tissues intervening between the maternal and foetal bloods in the fully formed placenta. Primitively there are six, the endothelium, connective tissue, and uterine epithelium of the mother and the trophoblast, mesenchyme and endothelium of the foetus. All these layers persist in the epitheliochorial placentae of pigs and horses, and only the uterine epithelium

disappears in the syndesmochorial placentae of cattle, sheep and goats. All the intervening maternal tissues disappear in man and the rodents, so that the maternal blood bathes the surface of the foetal tissues. The human placenta is classed, therefore, as haemochorial. Mossman (1926, 1937) has shown that in many rodents, including rabbits, guinea-pigs, rats and mice, the foetal capillaries project from the surface of the trophoblast into the lacunae of maternal blood, so that only the foetal capillary endothelium separates the two bloods at these points, and he distinguishes such placentae as haemoendothelial. The endotheliochorial placentae of carnivores occupy an intermediate position structurally, since the maternal endothelium as well as all the foetal tissues persist, but from the functional aspect, although it is known that the young of cats and dogs obtain antibodies through the colostrum, the evidence is somewhat conflicting as to whether they can pass the placentae, and it would be premature to conclude that they are physiologically intermediate. The view that the permeability of the placenta depends on the number of tissues intervening between the maternal and foetal blood streams fits the facts that antibodies do not pass epitheliochorial or syndesmochorial placentae and do pass in species which have haemochorial or haemoendothelial placentae. There are, however, several difficulties in the way of accepting the hypothesis as its stands. First, Kerr & Robertson (1943, 1947) have shown that the antigen of *Trichomonas foetus* is absorbed from the uterine lumen in cattle with the consequent production of active immunity in the blood, and that antibody can be demonstrated in the uterine secretion of animals sensitized in this way. Thus the very tissues, uterine connective tissue and endothelium, that are supposed to prevent the passage of antibodies from the maternal blood to the embryo in the syndesmochorial placenta of the cow, with the intact uterine epithelium in addition, permit the passage of antigen from the uterine lumen into the tissues and of antibody from the tissues into the lumen. Further, Barcroft, Danielli, Harper & Mitchell (1944) have shown that serum albumen marked with a disazo dye chemically attached to it by a diazo linkage can migrate at a rate greater than can be accounted for by diffusion through the mesenchyme forming Wharton's jelly in the umbilical cords of foetal sheep. They point out that Wharton's jelly is continuous with a similar tissue in the placental cotyledons. These observations, taken together, suggest that it is the trophoblast, and not the tissues between it and either the maternal or foetal blood, which is the barrier in ungulate placentae.

Moreover, the view that the passage of antibodies from mother to foetus depends on the number of tissues in the placenta intervening between the two blood streams involves the assumption that the allantochorionic placenta is the route. Little attention has been paid to the possibility that the yolk-sac might provide an alternative route in some mammals, notably the rodents. Yet Brunschwig (1927) has shown that the passage of sodium ferrocyanide and of ferric ammonium citrate into the embryo rat on the 9th day of development takes place through the yolk-sac, and not through the ectoplacental cone, which is the anlage of the allantochorionic placenta. Everett (1935), however, maintains that Reichert's membrane prevents the passage of ferric ammonium citrate into the yolk-sac of the rat, although it permits the passage of trypan blue. He claims that trypan blue reaches the

embryo rat from the maternal circulation through the yolk-sac placenta before it appears in the allantoic vessels. We have shown that antibodies pass in through the yolk-sac wall of embryo rabbits on the 7th and 8th days, together with all the other plasma proteins. It has not been possible, as yet, to test whether antibodies once within the yolk-sac cavity can pass from there into the foetal circulation, but there is no reason to suppose that they cannot. Since, in all the mammals mentioned above, the gut wall of the newborn young is known to be permeable to antibodies, this permeability rapidly declining after the first feed, it is reasonable to assume, in the absence of direct evidence, that the gut wall of the unborn young may be permeable also. The yolk-sac cavity is morphologically an extension of the lumen of the gut, with which it is in continuity, and its vascular splanchnic wall is histologically comparable to the gut wall. Hence it appears probable that antibodies could pass through the yolk-sac splanchnopleur into the vitelline circulation.

The bilaminar omphalopleur in the rabbit breaks down and disappears before the 15th day of gestation, thereby opening the yolk-sac cavity to the uterine lumen and leaving the entoderm of the inverted yolk-sac splanchnopleur exposed to the reformed uterine epithelium. Although the entoderm of the yolk-sac splanchnopleur is thereafter contiguous to, or even in contact with, the uterine epithelium, it is not in histological continuity with it, as is the bilaminar omphalopleur at earlier stages, since it does not possess the invasive properties of the trophoblast. Although we have demonstrated that antibodies pass into the embryo rabbit from the maternal circulation after the 15th day of gestation, no evidence is available* as to whether they do so through the yolk-sac splanchnopleur or through the allantochorionic placenta at this stage.

The yolk-sac in the human embryo, unlike those of the rodents, is separated from the chorion by the exocoele from the beginning, so that no omphalopleur is formed. Hence in man the allantochorionic placenta provides the only probable route into the embryo.

Since antibodies pass through the bilaminar omphalopleur in the rabbit, permeability to them is not limited to the allantochorionic placenta, but is rather a property of the trophoblast as a whole. The work of Kerr & Robertson (1943, 1947) and of Barcroft et al. (1944), referred to above, indicates that the trophoblast is the barrier to the passage of antibodies to the embryos in ruminants. It is well known that the trophoblast of man and the rodents possesses invasive or histolytic properties which the trophoblast of the ungulates lacks and we would suggest therefore, as a tentative working hypothesis, that the permeability of the embryonic membranes to proteins may be related to this property of the trophoblast, and not to the number of tissues intervening between the maternal and foetal tissues in the placenta.

* We have succeeded in demonstrating, since this paper was submitted for publication, that the passage of specific antibodies from the mother into the embryos of 23 to 25 days *post coitum* in rabbits takes place *via* the yolk-sac splanchnopleur alone and not in detectable amount through the allantochorionic placenta. An account of the technique employed, involving surgical interruption of the omphaloidean circulation of some, but not all, of the embryos *in utero*, and of the results obtained, is in preparation.

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Corrigenda

1. The blocks for *A* and *C* should be transposed but the letterpress against these blocks should remain as at present.

2. Letterpress for block *B*. *For* 'frog muscle (four fibres)' *read* 'frog muscle (two fibres)'.

The Royal Society and the Printer offer their apologies for the inconvenience caused to readers by this unfortunate error of transposition which was in no way the fault of the author of the article.

INSTRUCTIONS TO BINDER

A corrected leaf is attached which should be substituted for the original pages 515 and 516 when binding the volume.

sartorius muscle (1·6 m./sec. at 20° C, see Eccles *et al.* 1941). There are probably two reasons for this difference: first, one would suspect the isolated fibres to have a reduced 'safety margin' and therefore to conduct at an abnormally low rate (see Method). Secondly, the resistance on the outside of the fibres was very high (see § B 4), and this must cause a considerable drop in velocity (cf. Hodgkin 1939; Katz 1947*b*). For example, a 60*μ* fibre which conducted at a speed of 1 m./sec., had a ratio of outside:inside resistance of 1·02: the velocity of this fibre *in situ* would have been $1·0 \times \sqrt{[(r_i + r_o)/r_i]} = 1·42$ m./sec.

FIGURE 4. 'Voltage/current' relation at the polarizing electrode. *A*, from the experiment of figure 3*B*. *B*, from a M. extensor dig. IV. Ordinates· maximum amplitude of local potential change in mV. Abscissae· current strength in *μ*A Threshold of propagating spikes indicated by arrow. *Note.* In experiment B the deflexions were steady, while in A, with currents near threshold, the amplitude of the cathodic potential began to decline before the break of the current (cf. figure 3*B*).

B. *Electric constants of isolated muscle fibres*

It follows from these observations that the muscle membrane gives a local electric response in the region of the cathode which may persist for 100 msec. or more, and this causes the apparent membrane resistance to depart significantly from Ohm's law. To minimize this complication the analysis was restricted to the potential changes at the anode.

FIGURE 5. Anodic potential changes. A, M. adductor magnus, four fibres (diameters 70, 70, 45, 45μ). B, M. adductor magnus, two fibres (diameters 130 and 50μ). C, Carcinus nerve. Single fibre of 43μ outside diameter. Extrapolar distances shown in mm. Instant of current make is indicated by a dash. Time marks at 20 msec. interval.

The Lister Institute of Preventive Medicine

By A. N. Drury, F.R.S., *Director of the Institute*

[Plates 19 to 21]

On a Sunday morning early in 1889, the Lord Mayor of London, Sir James Whitehead, visited Pasteur in his institute in Paris. He went, as he says, as a business man to consult the records of the treatment for rabies that Pasteur had recently developed. He went, I believe, at the suggestion of his friend Ray Lankester who was much concerned at the increase of rabies in this country. He returned determined that some public recognition should be made in Great Britain of Pasteur's work for humanity and his generosity in treating, free of cost, persons who were bitten by rabid dogs in this country, and that the establishment of a treatment centre for rabies in London should be seriously entertained. He decided to call a meeting at the Mansion House, and a considerable correspondence passed between him and Ray Lankester as to the business which should be transacted. From the very outset Ray Lankester, supported by Sir Henry Roscoe, James Paget and others, was against the establishment in London of a treatment centre for rabies. They and many others had always argued that this disease could be stamped out from these islands by the muzzling of dogs and a proper quarantine for newly imported animals. Pasteur himself had recommended this line of action in the public press. Ray Lankester argued with the Lord Mayor that if a treatment centre was established in London it would only facilitate the evasion of the public duty to stamp out rabies. The possible establishment of a new institute had, however, got abroad and a vigorous press campaign was started, at the instigation of anti-vivisectionists, against this development, on the grounds that it would perpetuate in this country all the cruelty and pain which Pasteur had been accused of inflicting upon animals in Paris for many years past. The opposition was a very real one, for Ray Lankester wrote to the Lord Mayor just before the meeting: 'It will be necessary to have a good posse of police to guard the entrance, and the stewards must prevent anyone from obtaining admission who has not received an invitation.' The meeting passed off without incident. The business of the meeting concerned the public expression of thanks to Pasteur, the setting up of a committee to collect funds in order to make a presentation to his institute, and inviting the Government to stamp out rabies by the simultaneous muzzling of all dogs throughout the British Isles and by effective quarantine. Some of the speakers referred, however, to the idea of establishing a new institute. Michael Foster drew the attention of the meeting to the fact that those who pursued a certain branch of science 'are put upon a criminal footing and are only allowed to pursue their investigations upon ticket of leave', that 'it would not do simply to establish in this country a merely mechanical shop, so to speak, for the mere

repetition of inoculation. We are only beginning this great subject of inoculation; inquiry must go on, and unless an institute is kept sweet by the salt of investigation, it will become a hindrance.' He went on: 'I think that, with our present regulations, the necessary inquiries which belong to this work are better carried out in Paris than in London, and you would do well to give your money to Paris and not keep it for London ' Ray Lankester thought it was absurd to attempt to start an institute in London by means of private subscriptions. He said: 'It cannot be done, it is simply out of the question; it has been tried. But I may say I look forward to the time when an institute will be established in London in the only way in which it can be established, that is, under the auspices of the Government.'

In the autumn of 1889 the Mansion House Committee, under the chairmanship of the Lord Mayor, presented £2000 to the Pasteur Institute.

After the final meeting had been held, the Committee went to the Mayor's Parlour to take tea and the Lord Mayor expressed his sorrow that this was their last meeting. During tea-time Professor Roy, of the Department of Pathology, Cambridge, and Sydney Turner, the Secretary of the Mastiff Club, a dog lovers' society, expressed strongly the view that the idea of establishing an institute should not be given up. They convinced the other members of the Committee, such as Lister, Horsley, Roscoe, Ray Lankester, Thomas Huxley and Paget, that it might be possible, and the Mansion House Committee was dissolved and an Acting Committee immediately formed to carry this into effect. The Lord Mayor, through ill health, was Chairman of this Committee for a short time only, and he was succeeded by Sir Joseph Lister. The Committee immediately asked Roy and Turner to draw up a scheme and, within a few weeks, they presented a report to the main Committee suggesting that a Jenner-Pasteur Institute should be established in Cambridge and that the main objects should be 'the preparation and inoculation of material which has been found successful for preventive inoculation and the carrying out of investigations fitted to increase our knowledge of the nature of disease-producing germs'. They chose Cambridge, as they said, for the reason that the Institute would be affiliated to one of the largest and best-equipped pathological laboratories in England, that Cambridge was readily accessible from all parts of the country and was only 1¼ hours from London by train.

The first appeal to the public for funds was not made till December 1890. The appeal was for the establishment of a British Institute of Preventive Medicine, to be situated at Cambridge, the objects of which would be primarily the investigation of the various infective diseases of man and animals, and secondly, the preparation of protective and curative materials which are found to be of value. You will notice that the emphasis is now placed upon scientific investigation, whereas in the scheme suggested by Roy and Turner it was placed upon the preparation of curative material. This appeal was sent to scientific societies, such as the Royal Society, to all the city companies and to many private individuals. In addition, a person was appointed to make house to house collections. Money did not come in at all freely, but certain of the city companies, especially the Grocers' Company, responded generously. The individual responsible for the house

to house collection died only a short while ago, and he told how on one occasion he went to a physician in Harley Street who quickly showed his sympathy by writing a cheque for £100, but the next told him equally quickly that he did not approve of preventive medicine and thought that a good epidemic was much more satisfactory. Fortunately, at this time, a certain Mr Berridge had left a large sum of money for the furtherance of sanitary science, and the Committee were able to persuade the Trustees to give some £40,000 to the proposed Institute. This gift had a definite influence in the situation of the Institute, for the Berridge Trustees made it one of the conditions of their offer that the Institute should be placed in London, and, in spite of the fact that negotiations had been carried a long way with the University of Cambridge and any change of plan would place Roy in an awkward position, the Committee, after several acrimonious meetings, decided that the Institute should be established in London.

By the beginning of 1891 about £60,000 had been collected and the Committee decided to incorporate the Institute as a limited liability company with the omission of the word 'limited' in order to impress the public with the fact that the Institute was not established for the purpose of gain but for purely charitable and scientific objects. The President of the Board of Trade, Sir Michael Hicks Beach, at first declined to grant the application without giving any reason for his decision. He agreed later to receive a deputation, and some 500 influential men and scientists met him to put the case. It transpired that one of the main difficulties in his mind was that if he granted the application it might be construed as implying approval by the Board of Trade for experiments on living animals, or as affecting the exercise by the Secretary of State of his discretionary powers under the Cruelty to Animals Act of 1876. As a result of this meeting and certain further correspondence from the Committee, Sir Michael Hicks Beach finally gave his sanction, and the British Institute of Preventive Medicine was incorporated on 25 July 1891, a special paragraph being inserted in the articles stating that the sanction did not imply any approval by the Board of Trade of experiments on animals.

The Committee next considered how and where the work should be carried out. They obtained from the Duke of Westminster, on very generous terms, a site in Chelsea Bridge Road, but it was clear to the Committee that the new buildings would take some time to complete, and there was still the question as to whether they would be able to get the new buildings licensed for experiments on animals. They, therefore, looked around to see if there was any other organization with whom they could effect amalgamation. They came to the conclusion that the College of State Medicine, a private organization which was carrying on investigation into infective diseases in addition to teaching sanitary science, had aims that were not far removed from those of the proposed Institute, and they had a house which was already licensed for animal experiments.

Late in 1893 amalgamation with this College was effected. This provided immediate accommodation, and the transfer of the licence for animal experiments to a new building would be relatively easy.

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much the same manner as it was in 1893, and the only alteration to the frontage has been the insertion of a square window in place of two half-round windows on the ground floor (figure 1, plate 19).

The erection of the new buildings at Chelsea was being carried through with difficulty, for the residents, again at the instigation of the anti-vivisectionists, I believe, immediately lodged a petition with the Home Secretary against the buildings on the grounds that the new laboratories would be a danger to the neighbourhood; the bacteria might escape from the windows, the attendants from the Institute would be living near by and might bring disease into their homes, and persons with disease would be coming into the neighbourhood for treatment. All this would destroy the amenities of the area in which the Grosvenor Road and the Chelsea Embankment were situated and which formed a favourite Sunday evening promenade.

The Institute immediately arranged for a representative to interview the tenants nearby and give them any information they would wish to have about the Institute. He has left a very human document detailing his visits. Several of the tenants told him they would leave if the Institute was built, while the majority 'did not understand the question, did not want to, had not signed the petition, and did not want any information'. He finished by saying he had a chat with the soldiers in the guard room of the barracks nearby where they were totally indifferent, but 'I was offered an unlimited supply of animals for experimental purposes for a consideration'. All these difficulties were finally overcome, but the buildings were not completed until 1898, the delays being largely due to the difficulties of building on the waterlogged banks of the Thames (figure 2, plate 19). Scientific work commenced in the new buildings in July 1898, the licence for animal experiments granted to the College of State Medicine having been transferred to the new laboratories.

The Institute had always had in mind the preparation of therapeutic substances, and in order to carry this out they rented in 1894 a small farm from Sir William Perkin, the discoverer of aniline dyes, at Sudbury, near Harrow. This proved insufficient for their needs, and in 1902 they bought an estate of 25 acres at Elstree. This estate had been run as a racing stable, so that the only additional buildings required were for laboratory accommodation (figure 3, plate 20).

The Institute was then relying, as it has done during its whole career, on income from endowments and the profits on the sale of therapeutic substances. At first the financial situation was extremely difficult, as income was insufficient to meet current expenses. Losses were also encountered from the sale of therapeutic substances; capital had to be sold to meet expenses. It seemed that Ray Lankester's belief that such an Institute could not be run on private subscriptions might prove correct. In 1912, however, the late Lord Iveagh made a most generous gift of £250,000 to the Institute and, at about the same time, the sale of therapeutic substances began to show profit, and this, together with a few substantial bequests and donations, has ensured an income sufficient for a progressive, though modest, research institute.

One of these bequests brought about a change in the name of the Institute. The year 1896 was the centenary of the discovery of vaccination against smallpox by

Jenner, and a sum of money was collected and presented to the Institute as a memorial to a man 'whose work was of importance to the whole human race'. The Institute, in accepting the gift, agreed to change the name from that of the British Institute of Preventive Medicine to that of 'The Jenner Institute of Preventive Medicine'. A few years later, however, this had to be abandoned, as a commercial organization supplying smallpox vaccine had a prior claim to the name 'Jenner Institute'. The name was therefore changed to that of the 'Lister Institute of Preventive Medicine'. No happier name could have been chosen. Lister had interested himself in the project from the very beginning and had given unstintingly of his time and influence in its Foundation and development.

As if three names for an Institute were not sufficient, a proposal was put forward in 1920 to change its name once again to the 'Lister Institute for Medical Research'. Fortunately, from the point of view of historical simplicity, this proposal was defeated.

In the beginning the management of the Institute was in the hands of a Council of twenty-four persons, seventeen of whom were representatives of various learned and scientific bodies, the remaining seven being elected by the members of the Institute. The members were persons who either subscribed a certain sum to the Institute funds or were invited to become members by the Council. When Lord Iveagh made his gift, the management was placed in the hands of a Governing Body of seven members. Of this Governing Body, one member is elected by the Royal Society, three by Lord Iveagh, and three by the Council of the Institute, which continued to exist in its original form. The Council thus ceased to be a directly administrative body, but continued to exert an influence over the Institute's affairs through the representatives on the Governing Body. Many eminent men and scientists have given of their time as members of the Governing Body, and others as members of the Council, and among those who have helped us have been Lister, Roscoe, David Bruce, Burdon Sanderson, Michael Foster, Ernest Starling, William Osler, Rose Bradford, Leishman, Boycott and Bullock.

I have gone into the history of the foundation of this Institute in some detail. It shows how unsympathetic, if not hostile, was the atmosphere to scientific investigation involving animals in those days, only just over 50 years ago, and stresses how resolute and determined the members of the Committee must have been in carrying the matter through.

The Institute has been actively at work for about 50 years. In its early days, in addition to research, teaching was an important branch of the Institute's activities, but this was largely discontinued in 1901 when departments of bacteriology had been established generally throughout the country. Some 2000 papers have been published as a result of the researches in medical and allied subjects. A great majority of these show solid advances in their subjects, while some of them record striking discoveries. In addition, the Institute gave help to Governments and municipalities to implement their duties to the public health at a time when they were not ready to do this themselves, and this took up a great deal of the time and energy of the Institute. The character of the departments has, therefore, been fluid and elastic. Although the general background has been bacteriology,

experimental pathology and biochemistry, special researches have been undertaken from time to time in response to urgent needs. The scientific staff has always been small, seldom more than thirty, but the number of attached workers, including investigators from the dominions and elsewhere, has often nearly equalled that of the established staff.

In 1905 the Institute was admitted as a school of the University of London for the purpose of research, and this gave an opportunity for students to join the Institute and to proceed to higher research degrees of the University. Until the formation of the National Institute for Medical Research by the Medical Research Committee (later the Medical Research Council), the Lister Institute was the main training ground for those interested in preventive medicine. The list of those who have worked at the Institute is a long and most impressive one, and many have made fine contributions to their subjects; it includes a Nobel prizeman, many who have been honoured by election to the Fellowship of the Royal Society, and others who have received honours for their work on behalf of the Home or Colonial Governments.

As one reviews the work of the last 50 years, one is struck by the large number of subjects investigated. They range, as a late director has put it, from purpura haemorrhagica to the synthesis of vitamin B_1, from plague to the coagulation of proteins by heat and alcohol, from fermentation to the domestic habits of the body louse.

It is difficult in a short time to describe more than a few of the Institute's scientific activities or to mention more than a few of the scientists who have contributed to the progress of their subjects. It may be best to take up each department in turn and to outline some of the more interesting achievements.

When the Institute commenced work it consisted of two main units or departments, that concerned with bacteriology and that with the examination of drinking water. The Bacteriology Unit was first under the direction of Dr MacFadyen, a man whose ideas were ahead of his technical competence. He, together with Dr Rowland, was chiefly interested in an attempt to obtain the antigens from bacteria in as normal a condition as possible, which he attempted to do by grinding them up in liquid air.

There then followed a period under Dr George Dean, during which many solid advances were made.

In 1903, Dr Martin was appointed Director of the Institute and, largely as a result of his initiative, investigations were carried out on the spread of plague in India, under the Committee for the Investigation of Plague in India, on which the Institute was represented in addition to the Royal Society and the India Office. In 1905 the Director, Dr Martin, with two members of the staff, went to India to work in the Parel Laboratory, Bombay, and, in collaboration with members of the Indian Medical Service, carried through a fine piece of epidemiological research. These researches, which were continued over three years, succeeded in establishing as the carrier of the plague bacillus, the rat flea (*Xenopsylla cheopis*), which usually infests the wild rat (*Mus rattus*) in tropical and subtropical regions of the world. Later work carried out at the Institute in London demonstrated

the mechanism by means of which the flea, by a process of regurgitation, is able to inject a fresh human subject with bacilli cultivated in its stomach upon blood sucked previously from an infected rat or human being.

For a considerable period Ledingham was responsible for the direction of the Bacteriology Department. His work on trench fever and its minute causal 'rickettsia' and the technique developed in its study was a preliminary to useful work on the viruses of vaccinia and fowlpox. There is now no doubt that the so-called 'elementary bodies' which could be demonstrated under a high-power microscope represent at least one stage in the life of these viruses. Proof was forthcoming in the results of later work which showed the specific antigenic action of preparations of these elementary bodies when injected into horses.

Bacteria, being living organisms, are subject to variations and mutations. These are manifested by differences in the character of their growth on artificial media, their power to ferment various sugars, and also their virulence and susceptibility to make protective vaccines. Bacterial variation is a subject which engaged the attention of members of the bacteriological staff for many years, particularly of Sir Joseph Arkwright, Sir John Ledingham and Dr Penfold. The discovery that alteration in the shape of the colonies of many pathogenic bacteria was associated with alteration in virulence and immunizing value was an impetus to these studies and has afforded valuable results.

More recently, observations have been carried out on the typhoid bacillus. It was found that when different smooth strains of typhoid bacilli are compared with one another in regard to their sensitiveness to agglutination by an O antiserum, they differ very widely. This sensitivity is inversely related to the virulence of the strains. Later work showed that the virulence and inagglutinability are due to the presence of a special antigenic component called the Vi antigen. This work has had important repercussions on the detection of typhoid carriers and the preparation of prophylactic vaccines against typhoid. Ledingham was appointed Director on Charles Martin's retirement in 1931.

The formation of the Water Laboratory in 1894 arose from the fact that the Berridge Trustees, in making their bequest, stipulated that a certain sum should be expended on a laboratory for the chemical and bacteriological examination of drinking water and on an investigation of the value of treating sewage by a process devised by a Frenchman, Dr Hermite. This process involves the treatment of sewage with electrolyzed sea water, and a trial was actually carried out in 1894 at Worthing in Sussex, where the limitations of the method were clearly exposed. Routine work on the chemical and bacteriological examination of water was carried on for some years. In 1898, a new department was opened for the practical application of bacteriology in industrial and commercial processes. This was led by Dr Morris who was interested in fermentation, but when he left two years later the department was fused with the Water Laboratory to which Dr Harden had been appointed in 1897. As a result of Buchner's epoch-making discovery, announced at about the time of Harden's appointment, that the living cell was not essential to the process of fermentation and that cell-free yeast juice would cause the decomposition of sugar, Harden commenced his researches in this field.

His studies soon revealed that the fermentation enzyme, zymase, would not convert sugar into alcohol unless another substance which functioned as a co-ferment was present. He also observed that the presence of phosphate was also necessary if fermentation is to take place. The separation of the co-ferment was achieved by using a gelatin filter introduced by C. J. Martin for the separation of high-molecular colloids from crystalloids. The zymase was retained by the filter while the co-enzyme passed through. Neither component alone would give rise to the fermentation of sugar, whereas when the two were mixed fermentation occurred. Many years later the nature of co-ferment was established as adenine pyridine diphosphonucleotide by the work of Warburg and von Euler.

Harden's discovery was an outstanding contribution to biochemistry and provided a clue to many other biological phenomena, such as the biochemical aspects of molecular contraction and bacterial metabolism. For these contributions Harden shared the Nobel prize for chemistry with von Euler.

Robison became a member of the Biochemical Department in 1913, and for the first time came into contact with biochemical problems. He commenced work with his characteristic thoroughness and enthusiasm, and soon announced the discovery of a new hexose monophosphoric ester which was destined to play an important part in the later development of biochemistry. He investigated in extreme detail the products of alcoholic fermentation, and the skill and care with which his work was undertaken can be judged from the fact that six hexosephosphoric esters were discovered during the following years. Early in 1923 Robison discovered the enzyme, phosphatase, in the aqueous extract of bones of young, rapidly growing animals. He used the bone phosphatase as a biochemical reagent and opened up new techniques for determining the constitution of the sugar phosphoric esters. Robison's more biological work was a direct outcome of his investigations on alcoholic fermentation and, with the knowledge gained by the discovery of the enzyme phosphatase, allowed him to suggest a probable mechanism for the deposition of the calcium salts of bone. During the following years Robison developed a theory of bone formation and produced much valuable experimental evidence to support the view that the full calcifying mechanism consists of a complex enzyme system of which phosphatase is but one component. Robison's work on this subject is a rare example of beautifully planned research carried out with outstanding skill.

Subsequent work in the department developed along entirely different lines. Investigations to establish the chemical nature of certain bacterial antigens had started some years before. The outbreak of war in 1939 seemed reason enough for hurrying forward these studies which were largely concerned with the isolation and purification of the antigens of the dysentery and typhoid group of organisms. Although the antigenic preparations were not used on a large scale, a small group of volunteers was successfully immunized in 1942 with the isolated and purified O somatic antigen of Shiga's dysentery bacillus. Investigations aimed at increasing our knowledge of the fundamental processes involved in blood transfusion were also commenced. The human A, B and O blood group specific substances have been isolated and characterized, and the genetical aspects of these biochemical

investigations are now being studied. The role of the toxins of *Clostridium welchii* in gas gangrene has also been investigated, and the findings open up a new approach to the chemistry and pharmacology of bacterial toxins. Similarly, a thorough study of the chemical structure of the important bacteriostatic substance, gramicidin, has recently been undertaken and has revealed the evidence of a new type of structure which may be common to other labile molecules of biological importance.

These two departments have continued without interruption from the very beginning, other departments have had much shorter lives. Dr Barnard, whose later work on microscopy earned him such well-merited recognition, was in charge of a short-lived Photographic Department in 1900. Until he left in 1906 he worked on the phosphorescence of bacteria and the bactericidal action of light of different wave-lengths.

In 1900 the Department of Pathological Chemistry was formed under the direction of Dr Hedin. Dr Hedin was interested in the proteolytic enzymes to be found in organ juices, but the more striking investigations of this department commenced with the arrival of Leathes, who carried out his early work on the metabolism of fat. At that time Cathcart and Dakin were working in this laboratory. When Leathes left to take up another post in 1905, this department was merged into that of the Water Laboratory and became the Department of Biochemistry.

Charles Martin became Director in 1903, and, as one would expect from our knowledge of the man, there was progress in many different fields. Co-operating with John Haldane, Boycott and Lieut. Damant of the Navy, an investigation into the physiological adaptation to raised and lowered atmospheric pressure was carried through, using a pressure chamber which was the gift of Dr Ludwig Mond. The results defined the precautions necessary to prevent accidents during diving operations in the Navy. This was followed by work on a breathing apparatus for use in rescue work in mines.

In 1911, a young Pole, Casimir Funk, was engaged in an attempt to separate from yeast the substance which would prevent beriberi in man. For this substance he coined the word 'vitamin'—a word which pursues us from the cradle pretty well to the grave.

The occurrence of beriberi and scurvy in the troops abroad during the 1914–18 war created a further urgency for the study of these diseases. A team of workers undertook researches upon the distribution and properties of the anti-beriberi vitamin—now known as vitamin B_1 and the anti-scurvy vitamin—now known as vitamin C. This team developed into a nutrition department. After the war this team went with workers from the Medical Research Council to Vienna and stayed there for three years carrying out an extensive series of investigations into rickets and scurvy which had become prevalent in central Europe.

It was found that the indications for successful prevention and treatment obtained from the results of experimental work with animals were applicable to the diseases in human beings. The results of the trials carried out in the Hospital in Vienna showed that the disease in infants could be prevented or cured with certainty either by the administration of preformed vitamin D in cod-liver oil or

other foods, or by exposure to ultra-violet irradiation from artificial sources, and that the physiological reaction of these three agents was indistinguishable.

With the coming of the Second World War, the problem of selecting the most economical and nutritious flour for making bread became important, and the value of flour derived from different portions of the grain was extensively investigated. In addition, the stability of vitamins under different conditions of storage was determined.

In 1906 a Department of Protozoology developed, the University having asked the Institute to accommodate E. A. Minchin, Jodrell Professor of Zoology. His main work was the detailed and very complete study of the life cycle of *Trypanasoma lewisi* in rats and fleas. He gave set lectures on tropical diseases which many officers of the R.A.M.C. and I.M.S. attended. He was a man of wide culture and experience, fond of quoting his Virgil or his Milton in the laboratory. He died at the early age of 49, but the interest in protozoology has continued in the Institute and has recently gathered impetus in a study of *Trichomonas foetus*, and in an attempt to separate the antigens by biochemical and biophysical methods from mass cultures of the organism.

In 1909 the Governing Body decided that the validity of conclusions drawn from many inquiries in experimental medicine, as well as those regarding the practical value of prophylactic and curative treatment, must ultimately rest upon the statistical analysis of the results, and that statistical treatment would aid in defining the relative importance of different means whereby a disease is spread. They appointed Dr Major Greenwood to lead a statistical unit and G. Udny Yule accepted an honorary post. Dr Major Greenwood left the Institute in 1919 to go to the Ministry of Health, and finally became the first Professor of Epidemiology and Vital Statistics in the University of London. The Institute thus played an early part in introducing into scientific investigation the importance of the statistical treatment of the results and was happy in securing Professor Greenwood, who, in his own inimitable way, has stressed this development.

In 1911, a unit for entomological research was started under A. W. Bacot. He was then 45 years old, had no set scientific education, having been a city clerk for 27 years, with entomology as his intense hobby. He developed into a research worker of great originality and investigated the bionomics of the flea and the louse, and became a member of the Yellow Fever West African Commission. In 1922, he collaborated with Sir Joseph Arkwright in Egypt, in an investigation into typhus. They both contracted the disease and Bacot unfortunately died.

As early as 1894, Dr Macfadyen had started a collection of organisms of interest to medical men, brewers and chemists. As the collection was maintained in a very desultory manner, most workers preferred to obtain their cultures from Germany. With the onset of the 1914–18 war, this source was no longer available, and this emphasized the need for a well-maintained collection in this country. In 1919, the Medical Research Council, in association with the Institute, decided to form a National Collection of Type Cultures. This unit has been housed in the Institute and has developed a world-wide importance.

In 1936, the Department of Biophysics was initiated. A new building was erected and the expense of the initial major equipment, comprising the high-speed ultracentrifuge and equilibrium ultracentrifuge, was partly borne by the Rockefeller Foundation. A new Tisselius electrophoresis apparatus was erected in 1937. Taking advantage of the new techniques thus afforded, work on vaccinial bodies, antitoxins, pneumococcal polysaccharides and normal and pathological human sera was undertaken.

At the outset of the Second World War, the unit turned its attention to the preparation of a liquid blood plasma which would stand long storage and transport without deterioration, and a method of ether extraction was developed which gave promising results. It seemed evident at that time that the ether method had much wider possibilities, but owing to more urgent war needs, little further work was then carried out. Immediately after the war, a joint unit with the Medical Research Council was set up to exploit the possibilities of the ether method with a view to preparing the various fractions of human sera. The value of these was being demonstrated in the U.S.A. where they were being prepared by an alcohol precipitation method involving an elaborate set-up. The ether method of precipitation has developed most fruitfully. By varying the concentration of ether, the hydrogen-ion concentration, the ionic strength and the temperature, it is now possible to obtain, with modest laboratory equipment, fibrinogen, prothrombin, β- and γ-globulins in a high state of purity and in good yield from human plasma or serum. Two of these products, fibrinogen and thrombin, which is prepared from the precipitated prothrombin, when mixed together form a very solid clot, which can be used to keep the two ends of a severed nerve in close apposition when they are sutured together. The same mixture can be used to stick or fix skin grafts on to areas which have been denuded of skin through severe burning. If the fibrinogen is treated in the laboratory in a special manner, a sponge-like material, fibrin foam, can be produced; this, when moistened with thrombin, can be placed upon a bleeding area. The blood is firmly clotted in the sponge and the haemorrhage promptly arrested. There is no need to remove the sponge, for, being composed of a natural substance, it is slowly absorbed. This has been used extensively in brain surgery where access to bleeding points is often extremely difficult. The 'γ-globulin' fraction, so called, contains the natural antibodies against diseases, and it has already been used for the modification or prevention of measles. The β-globulins contain the blood group agglutinins, while the albumin left in solution can be used for transfusion or for certain special blood grouping tests. There is little doubt that, as further experience is obtained, methods such as this, which make use of solvents for fractionating protein mixtures, will have even wider application. At the same time the availability of the various fractions of plasma in a high state of purity has allowed the attack on more fundamental problems.

The character of this unit is rather unusual, as it is concerned not only with research but also with production. It is responsible for processing some 50,000 bottles of human plasma a year for transfusion. The fractionation methods are initially defined on a laboratory scale and immediately adapted to large-scale

production, so that the products may be made available to the medical profession. As all the products are issued in the dried state, the unit's production commitments are considerable, but this responsibility calls for a steady supply of human plasma and serum, and the research worker has readily available ample supplies of human material for his more fundamental investigations. All the products are issued free by the Ministry of Health to the medical profession and no profit is made by the unit; a very desirable feature when it is remembered that the basal material, human blood, comes from the voluntary blood donor.

The manufacture of therapeutic substances commenced in 1894. At that date reports were coming from abroad of the successful use of diphtheria antitoxin, and Dr Armand Ruffer, who was secretary to the Institute at the time and had worked with Pasteur in Paris, undertook its preparation. The Institute was then housed at Great Russell Street and had no accommodation for horses, so Dr Ruffer approached Charles Sherrington, the Professor Superintendent of the Brown Animal Institution, and asked him to stable the necessary horses. In the autumn of 1894, the first antiserum against diphtheria to be prepared in this country was available (figure 4, plate 21), and the clinical trials were immediately carried out at the Eastern Fever Hospital in Homerton. These were not the first cases to be treated, for Pasteur had sent over a supply to the Institute in June and cases were successfully treated in July. In addition, while the first horse was being immunized, a young nephew of Sherrington fell seriously ill with diphtheria. Sherrington immediately asked Ruffer if he could bleed the horse. Ruffer agreed, though he pointed out that it was not ripe for trial. Sherrington tells in a dramatic manner how he bled the horse by lantern light one Saturday evening at the Brown Institution and the next day took the serum and injected it into his nephew. Though seriously ill, the boy recovered. Sir Charles Sherrington was therefore the first person to use diphtheria antitoxin prepared in this country.

The manufacture of antisera was continued under Dr Todd and later Dr McConkey, and in 1900 a major effort was put into an attempt to produce an effective anti-plague serum. This was a difficult and dangerous undertaking, as live virulent plague bacilli had to be injected into the horses. The manufacture of antisera of many other kinds has been carried on, and an agreement was made with Allen and Hanbury in 1895, which still stands, by which they became agents for the sale of the therapeutic substances prepared by the Institute.

In 1898 the Local Government Board undertook the preparation of smallpox vaccine, renting rooms at the Institute in Chelsea for this purpose. Here they stayed until 1907, when the Lymph Establishment at Hendon, which the Government had erected, was completed. In 1905 the Institute itself undertook the preparation of smallpox vaccine. In the beginning, like all producers, they used calves, but during the First World War, the supply of these animals became difficult and Dr Green, who was in charge of the work, investigated the possibility of using sheep. Animal tests and the clinical experience gained from the use of sheep vaccine indicated that its value was as good as that of calf vaccine, and owing to the fact that these animals do not suffer from tuberculosis and are much more easily kept clean than calves, the Institute has used sheep ever since. In 1946, the Government decided to close their

Lymph Establishment at Hendon, and it was gratifying that they asked the Institute to take over the supply of vaccine lymph for the whole country.

During the First World War the Institute became heavily engaged in the preparation of typhoid and other vaccines for the armed forces, and when the war terminated a department was set up for the commercial supply of bacterial vaccines.

From 1898 to the present time the Institute has carried on as a private research institute. In 1913, a proposal was put forward which, if it had been accepted, would have brought the Lister Institute to a close. The Government were then considering the establishment of a new Department of Medical Research in accordance with the provisions in the National Insurance Act of 1911. The Governing Body of the Institute considered whether it might not be in the best interest of medical science to offer to the nation the organization and resources of the Institute as a nucleus of the Government's scheme. The proposal was hotly debated by the Governing Body and, after many meetings with the National Committee and the Treasury, they decided by 5 votes for to 2 against—the Chairman, Sir John Rose Bradford, resigning his chairmanship, as he was against the proposal—to put the proposal before the members of the Institute who have a considerable say in any major change of policy. A special meeting was held in November 1914, and although the majority of those present spoke in favour of the proposal, it was rejected on poll by 39 votes against to 32 for, the proxy votes apparently turning the scale.

I have endeavoured to give you a short history of the foundation of the Lister Institute and in brief outline some of its scientific achievements. It is never possible to give a balanced picture of a research institute's activities, one subject is chosen because it can be told in a few simple words, another because it has a wide practical application. These are not necessarily more important than many of the small investigations which in themselves are fruitless and not worth the telling, but which have set other workers thinking and from which important lines have developed. As an example, Dr Harden in his Nobel Prize address, states that his discovery that phosphate plays an important part in alcoholic fermentation arose out of an attempt, which in point of fact proved fruitless, by Dr Macfadyen, a bacteriologist, to prepare an antizymase by injecting Buchner's yeast juice into animals.

The Lister Institute is an unusual institute. It is a private research institution and a school of the University of London. It derives its income from endowments, donations and the sale of therapeutic substances. It receives no subvention from the State or from the University of London. It has occupied rather an unusual position. Formed at a time when there were no other institutes of similar character, it not only carried out research work but became the adviser of the armed services and many Government Departments. This position naturally changed completely when the Government set up the Medical Research Council with its own Institute for Medical Research. Since that date the Institute has maintained very close connexion with the Medical Research Council, and joint units between the Council and the Institute have been established and the Council has housed, and still houses, some of its special units in the Institute's buildings.

I think it is clear that the history is one of which the Institute may be justly proud. It could be argued that it was formed at a time when many matters of scientific interest were ripe for development, and that no group of scientists could have failed to achieve fine results. I think it is more likely due to the fact that the Institute has been extremely fortunate in all those who have given time to its direction and to the quality of the scientists it has attracted to its staff. As one studies the lives of the early members of the Institute, one is struck by their enthusiasm and enterprise, working in fields that, owing to the state of knowledge at the time, were extremely dangerous; they pushed ahead with unpleasant and sometimes fatal consequences to themselves and their assistants. A tradition of honest work and co-operation was quickly built up, with no admission of secrecy. If this tradition can be maintained, the Institute should continue to play an important, though modest, part in the field of preventive medicine; a field into which such an intensity of effort is being put to-day that could not fail to give satisfaction, if not astonishment, to those who laboured so earnestly in 1889 for the foundation of the Lister Institute.

DESCRIPTION OF PLATES 19 TO 21

PLATE 19

FIGURE 1. 101 Great Russell Street The house belonged to the College of State Medicine with which the British Institute of Preventive Medicine amalgamated in 1893. Scientific work was carried out in this building from 1894 to 1898.

FIGURE 2. The laboratories in Chelsea Bridge Road, London The right-hand portion was completed in 1898 and the part to the left was added in 1910.

PLATE 20

FIGURE 3. The laboratories at Elstree, Herts. The Departments of Vaccine Lymph and Bacterial Vaccines are on the left, while the Serum Department and stables are in the centre and right respectively.

PLATE 21

FIGURE 4. The horse 'Tom'. The first horse in Great Britain to be immunized against diphtheria. Dr Ruffer is standing on the left and Mr Robertson, the veterinary surgeon, in the centre. It was from this horse that Sir Charles Sherrington obtained the serum with which he treated his nephew. (From an old photograph in possession of the Institute.)

FIGURE 1

FIGURE 2
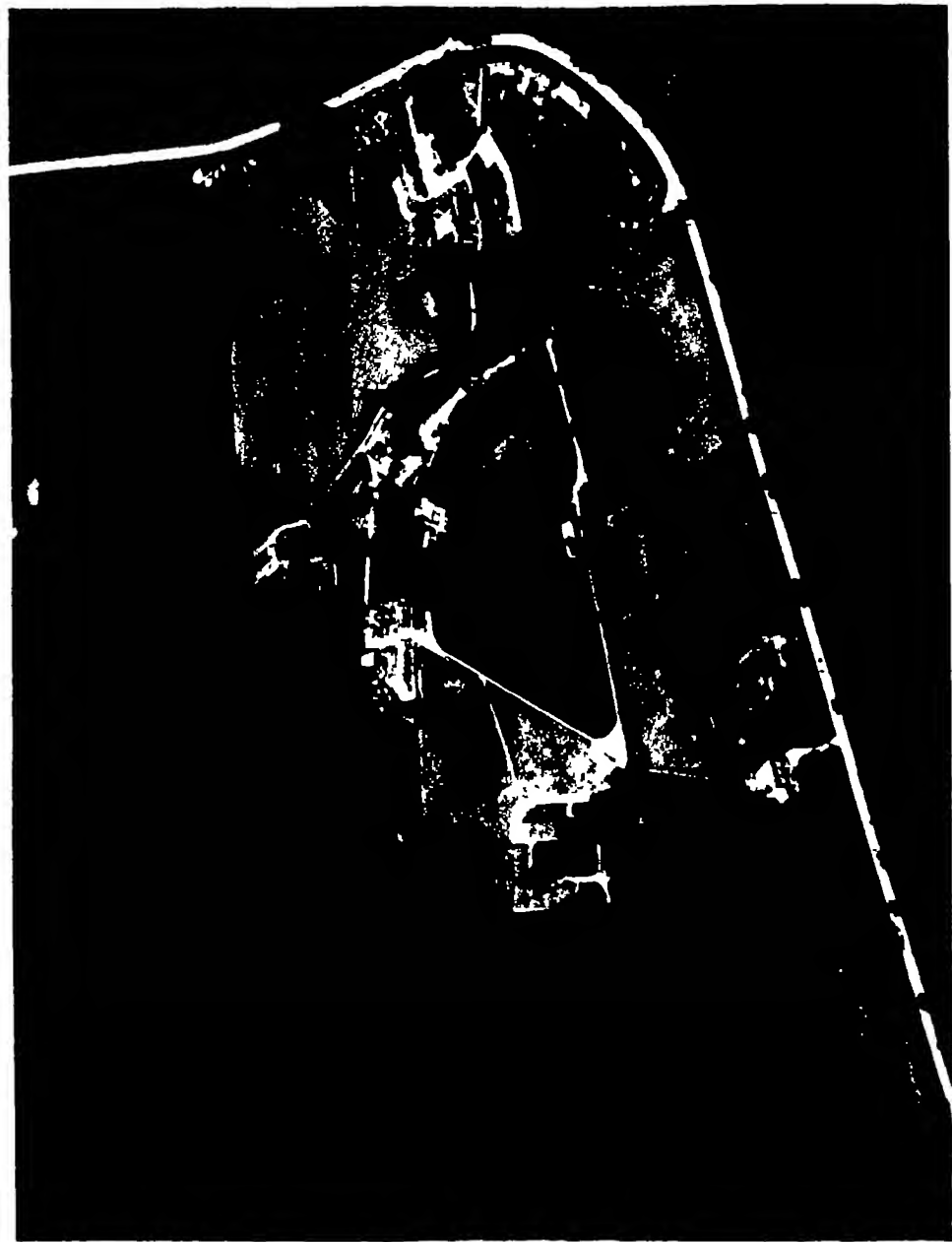
FIGURE 3

The Royal Botanic Gardens, Kew

By Sir Edward Salisbury, Sec.R.S.

Science can be defined as the philosophical co-ordination of classified information. Accurate identification of the units to be classified is fundamental to all scientific progress, and the Royal Botanic Gardens, Kew, has as its main function this service to science with respect to plants. By the public generally the Institution is usually regarded merely as an exceptionally beautiful garden and a pleasant resort, because these by-products of its equipment as a research organization are far more conspicuous than those provisions which are more directly concerned with its serious purposes.

It will help to place those purposes in true perspective if we review briefly the origin and history of the Institution. Although it is little more than a century since Kew became a National Research Establishment, the development at Kew of a Botanical Garden was the conception of that remarkable woman Princess Augusta, the mother of George III. Thus it was the enterprise and initiative of this individual in her private capacity, establishing an unusual type of garden on her own property, which explains why the largest botanical collections of living plants in the world are located on a rather sterile sandy soil, that from the point of view of culture has many defects and few merits. Sir William Chambers, writing in 1763, alludes to this fact when he says of Kew Gardens, 'what was once a desert is now an Eden. The judgment with which art hath been employed to supply the defects of nature and to cover its deformities hath very justly gained universal admiration.' However, in the days when labour was cheap and farm-yard manure plentiful, the building up of soil fertility was no hard task. But the impression of a favoured area which visitors to Kew often carry away is a tribute to generations of skilled cultivators whose superb craftsmanship has minimized the intrinsic defects of the soil and the pollution of an atmosphere laden with soot and sulphur dioxide. Thus only thirty years after Princess Augusta began the project Erasmus Darwin (1791) could write, 'So sits enthroned in vegetable pride Imperial Kew by Thames' glittering side.' There are a number of botanical gardens as distinct from Physic Gardens, far older than Kew, such, for example, as those at Padua, and Montpellier, but Princess Augusta when she began to create her garden in 1759 was something of a pioneer in that she collected plants for their own sake, and not merely because they were useful in medicine, or had other economic assets. She was assisted in this task by the Third Earl of Bute, of whom a contemporary wrote that 'he was unfitted to be Prime Minister on three counts, firstly because he was a Scotsman, secondly because he was a friend of the King and thirdly because he was an Honest man'. But, however unfitted he was as

a politician, he possessed undoubted ability as a botanist and was in effect the first Director of the Gardens.

The total area after 20 years was only 9 acres, but from the list published by the Curator Aiton (1789) we see that there were already in cultivation no less than 5500 species, and the garden had in fact already attained an international reputation. It was not till 1840 that the garden changed from private to public ownership, largely through the report of a Fellow of this Society, John Lindley, then Quain Professor of Botany at University College, London, and also through the powerful influence of the Sixth Duke of Bedford. With the death of the Princess Augusta in 1772, the control of the botanical garden passed to George III, who shared his mother's predilections. He combined the gardens of Kew with those of Richmond Lodge and hence the employment of the plural ever since, although the area devoted to the botanical collections, as distinct from pleasure gardens, was still only about 15 acres when the gardens were taken over by the nation. Of more immediate importance was that George III invoked the help of Sir Joseph Banks, who for a period of 47 years exercised scientific oversight of the Gardens, so that the association of the Royal Botanic Gardens and the Royal Society has been close from its inception, since the scientific direction has always been in the hands of a Fellow. Banks was President of the Royal Society for over 40 years during his unofficial directorship of the Gardens, and the second official Director, Sir Joseph Hooker, was President of the Royal Society from 1872 to 1877, whilst Sir David Prain was Treasurer from 1919 to 1929. So that by a happy coincidence, of the eight Directors of Kew, actual as well as nominal, no less than four have served as Officers of the Royal Society, totalling 58 years of such association.

When the Gardens were handed over to the public in 1841, Sir William Hooker was appointed Director, the first in name as well as in fact, and the much needed enlargements were made   Between 1842 and 1846 the Gardens were extended to almost their present limits, the only recent addition being the gift of the Queen's Cottage grounds in 1897 by Queen Victoria. The total area is now about 300 acres with a pinetum of some 50 acres at Bedgebury in Kent, where conifers can be grown free from the atmospheric pollution so detrimental to their welfare at Kew.

Kew Gardens, originally under the Board of Works, was placed under the Ministry of Agriculture and Fisheries in 1903, and financed by a block grant, the cost of maintenance now being of the order of £95,000 per annum. The Director of Kew is also Botanical Adviser to H.M. Government, and as such is called upon to advise and make recommendations on numerous technical and economic questions, so that the Director's office staff includes, in addition to an Assistant Director, an Economic Botanist, a position at present held by Sir Geoffrey Evans.

The total staff numbers about 270, of which over half are concerned directly with the culture of the living collections. The scientific staff numbers about 40. The remaining staff includes 40 constables necessitated by the admission of the public. It is important to realize, however, that in addition to the scientific staff itself a considerable number of visiting scientists are normally working in the Institution, and the striking tributes paid at the time of the centenary bear witness to the international service which the facilities at Kew afford.

The main work of the Institution is organized into four Departments, namely, the Herbarium and Library, the Museums, the Jodrell Laboratory and the Living Collections.

The Herbarium and Library occupy the largest Department in respect to buildings, since they together constitute a storehouse of specimens and literature unrivalled both in quality and quantity for purposes of taxonomic research. The oldest portion of the Herbarium was once the residence of the Duke of Cumberland, who later became King of Hanover, and was therefore known as Hanover House. The aim of Kew Herbarium is to provide international science with an extensive collection, particularly of the flowering plants of the whole world, and to this end we already possess between five and six million herbarium specimens including more than 200,000 types, using the term type in the broad sense. A better idea of the magnitude of the collection is perhaps gained by stating that to go through the Herbarium carefully and note the number of types would occupy one person, without holidays, rather more than 40 years of continuous work. Since any increase of knowledge usually involves reference to the type specimen, such can, of course, never be discarded, and the Herbarium building has in fact been enlarged on no less than three occasions, the last addition being in 1932. One of the problems that has to be faced is the proper control over such expansion of collections and the need for avoiding unnecessary overlap between the functions of different herbaria, since the mere housing and care of specimens makes a serious claim on space, personnel and public funds. The average number of specimens received by Kew each year is in fact about 50,000. All additions to the collections are carefully fumigated before taking their place in the cabinets, and, indeed, it is contrary to regulations for any specimen to be brought into the Herbarium without such disinfection. The cabinets are arranged according to the Bentham and Hooker system, and the specimens of any one species are grouped according to the geographical provenance of the material. Since the Kew collections are extensive, embracing the flora of the whole world, they are necessarily only intensive in respect to certain very restricted groups, and it may be hoped that other national herbaria, both at home and abroad, will accept the responsibility of making intensive collections of particular taxonomic groups, or local areas, since only by such division of labour, in the acceptance of responsibilities, can the requisite scientific provision for taxonomic research be effectively made without undue strain on the resources of individual institutions. Moreover, unless duplication of function be avoided the charge on public funds cannot be justified.

The Herbarium is in charge of the Keeper, under whom there is a staff of qualified taxonomists with Experimental Officers and Scientific Assistants. Each of the Principal Scientific Officers is responsible for a geographical area and tends to specialize on families characteristic of that region. This geographical basis for subdivision of the work has been found of more practical convenience than a strictly taxonomic subdivision which might seem more logical It is thus possible to ensure that the officer in charge of an area has a personal knowledge of the region concerned and is acquainted with the plants as living organisms and not merely as dried specimens. Moreover, the regional basis facilitates the establish-

ment of personal contacts of the officer with the taxonomists and collectors of the region for which he is responsible. The only exceptions to the geographical basis are first the grasses, a group which because of its large size (there are at least 10,000 species) and its great economic importance is the sole charge of a Principal Scientific Officer. Similarly, the ferns, the fungi and other cryptogams are also allocated on a taxonomic and not a geographical basis. The geographical sub-divisions employed are Europe, the Mediterranean and North Africa; South Africa; Tropical Africa; North Asia, China and Japan, India; Australasia; North America and South America.

In addition, the South African and Indian Governments support assistants for their respective areas who benefit by the training, advice and facilities which Kew can offer and familiarize themselves with the type material, whilst, on the other hand, their acquaintance with the living flora and vegetation often enables them to make reciprocal contributions We have every hope that this type of liaison will extend to other Dominions and Colonies to the benefit of themselves and Kew, and arrangements have been made with this objective as soon as the requisite personnel is available.

To Kew Herbarium come scientists from all over the world, and it is rarely that there are not taxonomists from a diversity of foreign lands working with the collections for periods ranging from weeks to months and, indeed, many of the most distinguished systematic botanists of the world have visited Kew and paid tribute to the facilities which it provides.

Apart from the preparation of monographs and the description of new species much of the time of specialists is devoted to the identification of specimens sent in by botanists, especially from various parts of the Commonwealth, and to the answering of inquiries During the recent war, most of the scientific staff of the Herbarium were either seconded for work more immediately connected with the war effort or had to take charge of the collections evacuated to areas where such inflammable and irreplaceable material was less liable to damage. As a con-sequence of this and the retention of specimens by collectors abroad till the end of hostilities we have now some 125,000 specimens representing accumulated arrears Much of the identification of such collections serves as a foundation for the preparation of regional floras to which Kew has made a notable contribution. Some thirty major floras and monographs have been prepared at Kew, including such monumental works as Bentham's *Flora Australiensis*, the *Flora Capensis* and the *Flora of British India*. Each of these three works consisted of seven volumes, and the last contained descriptions of over 15,000 species The ten volumes of the *Flora of Tropical Africa*, in which some 18,000 species were dealt with, and the classical floras by Hooker of New Zealand and Antarctica indicate the magnitude of these contributions The last of such floras to be produced was that of Hutchinson and Dalziel for West Tropical Africa just prior to the last world war. The prepara-tion of the much-needed *Flora of East Africa* has already begun, although the extended area and vast number of species with which this will deal involves a task of such magnitude that unless considerable additional assistance is forthcoming it may well be some years before much progress can be made.

The unrivalled taxonomic Library of some 45,000 volumes is an indispensable part of the equipment which, in association with the Herbarium, enables Kew to perform efficiently another international service for science, namely, the production of the *Index Kewensis* This is a catalogue, published by the Oxford University Press, of the Latin names of all the flowering plants which have been properly described since 1753, giving references to the original description and recently citation of an illustration if such exists. Begun in 1893 it contained over 375,000 citations, and the project was made possible by a gift from Charles Darwin bestowed as a mark of gratitude for the help he had received from Kew. A *Supplement* has been produced each quinquennium since then, and the work thus provides an indispensable tool for students of plant classification throughout the world.

The *Icones Plantarum* is a periodical published at Kew with the aid of a private bequest, the Bentham-Moxon Trust. It provides figures and descriptions of new and rare species prepared by the Kew staff and issued periodically. In this way since its inception illustrations and full botanical descriptions have been furnished of 3475 species. The Bentham-Moxon Fund, which provides this and a number of other botanical services and amenities, is a bequest with an income of about £1400 vested in trustees of whom the Director is ex-officio chairman and the Keeper of the Herbarium is also a member. This trust, which is free of the controls indispensable from Government funds, is of the greatest value, and double this sum could be spent with advantage The trust was established in 1885 under the will of one of our Fellows, George Bentham, and was greatly augmented by the legacy of Mr and Mrs Moxon It is due to this fund that Kew has acquired *inter alia* some invaluable collections of botanical illustrations, including many by famous artists such as those of Jacquin and Ehret.

Another publication to which reference must be made is the *Botanical Magazine*, for though published by the Royal Horticultural Society, the material is all prepared and the work edited in the Herbarium at Kew. Publication was begun by Curtis in 1787, and from that time has furnished admirable coloured illustrations and descriptions of considerably more than 8000 species Last but not least, the *Kew Bulletin* furnishes a medium for publication of miscellaneous botanical contributions amongst which descriptions of new species, critical notes, and articles concerning economic and geographic botany, are conspicuous features.

Some idea of Kew's contribution to taxonomy can be gained from the fact that during the first 30 years from 1841 to 1870 there were produced about 460 papers, but during the next 30 years the number of scientific contributions had increased to 1400 and over 50 publications ranging from descriptions of single species to substantial volumes have been produced annually since that time.

The Living Collections supplement the information provided by the dried herbarium specimens. The growing plants are under the general care of a Curator with Assistant Curators in charge of each of the main subdivisions. These are· (1) the arboretum and shrub collections; (2) the rock garden, aquatic garden and herbaceous beds; (3) the tropical, orchid and economic houses, (4) the temperate house; and (5) the decorative department.

It is important to appreciate that some 45,000 species of plants are in cultivation at Kew, and that many of these are annuals that require to be raised each year, often from seed which involves precaution against hybridization. The arboretum presents many problems, not the least of which is that no systematic provision has been made in the past to ensure that as trees become old and decrepit there are younger individuals to take their place. The fact that even the longest-lived trees at Kew appear not to be able to remain healthy more than about 200 years adds to the difficulty of securing adequate successional planting. Most of the trees were planted without any such considerations and hence of many species there is but one individual, whereas of others, such as the evergreen oak, there are far more than the space available can justify. It is, indeed, the very limited planting area now available that is the obstacle in attempting to remedy an obvious defect in a collection where planting should be not for any particular generation but for posterity also. Only a considerable extension of the Gardens would enable the requisite succession to be fully provided for.

The arrangement of the living collections in the open has in the past been mainly taxonomic. This has, of course, certain obvious advantages in regard to the proximity of allied species and as facilitating the search for a particular plant in this vast assemblage. But such advantages, though not inconsiderable, may, especially on a poor soil such as that at Kew, be outweighed by the circumstances imposed of growing in the same conditions species which, however closely allied, may in fact demand very different types of soil, degrees of shade, shelter and drainage. For this reason no attempt is made in either the rock garden or the aquatic garden to group plants on a taxonomic basis, and quite recently a chalk garden has been added to the ecological grouping which it is hoped to extend both in the interest of scientific value and cultural efficiency.

The living specimens of trees and shrubs in the arboretum which at the beginning of the century numbered 4500 now comprise some 10,000 species. They are an even more necessary complement to the *hortus siccus* than living specimens of smaller species in respect to which latter even a dried specimen may convey an approximation with respect to growth and habit. The tree collections provide useful information to the foresters and horticulturists, whilst here and in other sections the growing plants are a source of material for purposes of research.

Amongst the more notable trees at Kew may be mentioned the male maidenhair tree (*Gingko biloba*) which probably dates from about the time of Princess Augusta. There is a very beautiful example of the chestnut-leaved oak (*Quercus castanae-folia*), a species that exemplifies discontinuous distribution very strikingly, being found wild only in the Caucasus and Algeria. The planes are represented by two superb specimens, one the oriental plane (*Platanus orientalis*) near Museum III, and the other in the rhododendron dell is the London plane, a hybrid between the former species and *P. occidentalis*. The oldest trees in the gardens are a decrepit *Robinia pseudacacia*, and a persimmon, *Diospyros virginiana*, probably the finest in Britain, both planted in 1762, and a *Sophora japonica* planted 9 years earlier, whilst there is a magnificent wistaria that may well be even more aged. Amongst recent additions to the arboretum mention may be made of seedlings of *Meta-*

sequoia glyptostroboides, a close ally of the redwood, chiefly interesting because of its extremely local occurrence in China, in an area of some 300 square miles in the Szechuan province and with a fossil lineage that goes back some hundred million years, so that like *Gingko biloba* it can be termed not inaptly a living fossil.

One feature of physiological interest presented by Kew is the growth of self-sown trees upon its old walls. Thus on the wall of Cambridge Cottage can be seen a *Thuja orientalis* over 50 years of age (see plate 24), the only nourishment for which is derived from the bricks and mortar in which it is rooted. Other species that persist upon the same meagre rations are *Buddleia variabilis, Laburnum vulgaris, Pinus austraiaca,* a pear tree and a lilac.

The herbaceous collections are necessarily in much the greater part composed of exotic species. But it is curious that hitherto no serious attempt has been made to grow the rarer and the scientifically more important native species, a serious gap that a perusal of the seed exchange lists of other botanical gardens will show is shared by most others and has in the past been a not infrequent handicap to autecological studies. Steps are being taken to remedy this defect.

The temperate house is the largest glasshouse in the world, an eighth of a mile from end to end and contains about 2000 species belonging to 561 genera. It is here that the plants of Australia and New Zealand are chiefly housed as well as the more tender rhododendrons. The collection of tree ferns from New Zealand is a triumph of technical skill and horticultural grouping. The Australasian flora is notoriously difficult to maintain in health in this climate, but thanks to the remarkable skill of Mr Raffill, who has been in charge for many years, we have not only achieved success with a number of extremely rare and difficult subjects, but thanks to the remarkable collections of seed made by Captain and Mrs McEachern during the war years, Mr Raffill has now such a wealth of new material that the provision of an additional temperate house will be essential, and I am glad to be able to state that approval has been given for its erection.

The next largest house is the 60 ft. high palm house designed by Decimus Burton, where the collections of greatest scientific interest are the cycads, of which we have representatives of most genera.

The extensive range of houses near the chalk garden provides for the growth of a variety of economic plants which have frequently provided valuable research material for biochemical and pharmacological investigations Here, too, is housed that remarkable water lily, *Victoria regia,* which, despite its large size, a single plant occupying almost the whole of a 36 ft tank, is a comparatively short-lived annual that compasses its entire life cycle in the space of 9 months, sometimes growing at the rate of more than 2 in. in a night.

Collections of insectivorous plants, pelargoniums and orchids are provided for in adjacent houses. The orchid collection, thanks to the generous gift of the executors of the late Sir Jeremiah Coleman, now comprises a remarkable assemblage of species probably without parallel, though still lacking representation of many terrestrial genera. Other houses contain a noteworthy collection of succulents where the pebble-like *Lithops* provide unrivalled examples of plant mimicry, whilst some of the cacti and succulent euphorbias provide subjects as difficult as

any to cultivate in the moisture-laden atmosphere polluted by the proximity of a gasworks. The succulent collection was recently augmented by valuable additions of numerous species presented by Her Majesty and by the Succulent Society of South Africa. In the tropical fern house can be seen excellent examples of the ancient groups of the Marattiaceae and Osmundaceae with an extensive collection of pteridophytes from the giant cibotiums with fronds 15 ft. in length to the small *Selaginella apus*, chiefly noteworthy for being a living cryptogam that has adopted the seed habit. Other houses are devoted to a magnificent display of filmy ferns augmented this year by the gift of the late Mr Adney's collection, to tropical Araceae and to decorative species, the last named being for the benefit of the general public more than the scientist.

The care of the living collections affords a means of training in advanced horticulture, and to this end the permanent gardening staff is augmented by some forty student gardeners recruited solely from those who have already had at least 4 years of approved gardening experience in a first-class establishment. Here we may recall that William Cobbett was amongst those who have worked at Kew. The value of this training is evident from the fact that a large proportion of important horticultural posts both here and abroad are occupied by men who hold the Kew certificate.

No mention of the living collections would be complete without reference to the role Kew has played in the distribution and establishment of economic species in various parts of the Empire. Prior to the advent of air travel the development at Kew of the travelling greenhouse, known as a Wardian case, made the transport of living material over long distances possible. Hence Kew's success in establishing the plantation rubber industry and distribution through the Commonwealth of such economic plants as cinchona, coffee, pineapples, bread fruit, aleurites and many others. Moreover, as with the hybrid bananas from Trinidad, Kew has served as a house of quarantine, since the transport of material without such growth under expert supervision may be attended with grave risks, particularly with regard to the introduction of virus diseases. Mention should also be made of the extensive exchange of seeds with other scientific institutions and individual investigations. The last seed exchange list just issued enumerated more than 2500 species, and last year 7890 packets of seed were distributed to various parts of the world. Material for purposes of research was also supplied to many Universities and other research departments.

Four Museums serve to house the economic collections. They perform a dual role both as storehouses for research material and also as an instrument of education. The most noteworthy as a building is Museum No. III, constructed as an orangery. It is a monument to the genius of its designer, Sir William Chambers, and was built in 1761. Not least of the value of these collections of fruits, seeds, timbers and plant products of every kind, from crude drugs to spices, is for purposes of comparison in relation to the innumerable requests for identification which almost every post furnishes.

It was in relation to such inquiries that the fourth Department, the Jodrell Laboratory, developed its present functions. Here the main emphasis is on plant

anatomy, especially studied as a taxonomic character. Some 40 years ago the late Dr Boodle, then Keeper of the Jodrell Laboratory, translated, with Professor Fritsch, Solereder's *Systematic Anatomy of Dicotyledons*. Since that time the material examined at Kew and the knowledge acquired particularly from the living collections has been so great that the present Keeper, Dr Metcalfe, has, in collaboration with Dr Chalk, produced a second edition that will be substantially a new work. Plans have been made for a sequel to follow in due course which will treat of monocotyledons.

In this connexion it is of interest to recall that Scott carried out much of his fundamental work on fossil plants in the Jodrell Laboratory, and that here, too, Brown and Escombe conducted their classical researches on diffusion. Since that time the Jodrell has always served as a laboratory where anatomists, physiologists and cytologists have carried out researches on the living material that the gardens so richly provide

The inquiries which arrive by every post number over 10,000 in the course of a year. Some of these are relatively unimportant and are quickly dealt with, but others often demand research, sometimes of an extensive character. Specimens sent by some overseas taxonomist for precise identification may even involve the revision of a genus, whilst meticulous microscopic examination of fragmentary material from a suspected person's clothes, upon which life or death can depend, represent an appreciable investigation.

It may not be inappropriate here to refer to the relation of the public generally to the Royal Botanic Gardens. More visitors come than ever before. Last year the total was over a million and a half and 75,000 have been known on a single day. A by no means negligible proportion of these are genuinely interested in the collections from the botanical, horticultural or arboricultural points of view; such use the gardens for their proper purpose. A great majority come for sheer aesthetic pleasure; they, too, are welcome, but a minority know nothing and care less for the real purpose of the Gardens, which they regard as a public park, only differing from others in the restrictions, imposed to safeguard the collections, which they often resent and sometimes disregard. Such are both an incubus and a menace to the valuable treasures which it is Kew's privilege to nurture for the public weal. The best safeguard is an informed opinion, which Fellows of this Society can do much to foster, so that I am grateful for the opportunity afforded to make better known the priceless treasures that it is our good fortune to possess, our duty to cherish and obligation to develop for future generations.

The following is a list of the floras and monographs only, prepared at Kew and published as separate works, i.e. not in periodicals or other publications:

Baker, J. G. 1877 *Flora of Mauritius and Seychelles.*
Baker, J. G. 1887 *Handbook of the Fern Allies.*
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Baker, J. G. 1889 *Handbook of the Bromeliaceae.*
Baker, J. G. 1892 *Handbook of the Irideae.*
Bentham, G. 1863–78 *Flora Australiensis*, vols. 1–7.
Bentham, G. 1861 *Flora Hongkongensis.*
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Bentham, G. 1904 *Handbook of the British Flora*, 8th ed., revised by Sir J. D. Hooker.

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Duthie, J. F. 1903–29 *Flora of the Upper Gangetic Plain*, vols. 1–3.

Fitch, W. H. & Smith W. G. 1905 *Illustrations of the British Flora*, 6th ed.

Grisebach, A. H. R. 1859–64 *Flora of the British West Indian Islands*.

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Massee, G. E. 1891 *British fungi: Phycomycetes and Ustilaginaceae*.

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Trimen, H. & Hooker, Sir J. D. 1893–1900 *A handbook of the flora of Ceylon*, vols. 1–5.

Snowden, J. H. 1936 *The cultivated races of* Sorghum.

For a complete list of publications prepared at Kew from the year 1841 to 1906, see *Kew Bulletin*, 1897, pp. 2–84, 238–240, 1905, pp. 1–8; 1907, Append. v, pp. 96–152.

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DESCRIPTION OF PLATES 22 TO 24

PLATE 22

Aerial view of Kew Gardens

PLATE 23

Upper Sir William Chamber's Orangery, now Museum no. III, built in 1761.
Lower The interior of the extension of the most recent Herbarium.

PLATE 24

Upper *Thuja orientalis* over 50 years of age, rooted in wall only.
Lower *Victoria regia* in flower. Despite its size this flower is an annual.

Director

Assistant Director

Economic Botanist

Policy; General Direction of Establishment; Correspondence and relationships with Government Departments and Botanical Institutions; Public Relations; Constabulary and Attendants; Establishment matters; Publications; Education of Student Gardeners

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The insect as a medium for the study of physiology

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INTRODUCTION

Insects live and feed, move, grow and multiply like other animals; but they are so varied in form, so rich in species, and adapted to such diverse conditions of life that they afford unrivalled opportunities for physiological study. The general problems of physiology are much the same in all groups of animals; and this lecture, which represents, in effect, an apology for the study of insect physiology, is an attempt to show that among the insects may be found material well suited for the solution of many of these problems.

By human standards most insects are small in size, and this brings with it certain features which dominate their physiology. There is the same degree of functional specialization in their organs as there is in mammals; but they are made up of cells of the same dimensions as those in other animals—often, indeed, of cells which are larger than most Each organ therefore contains far fewer cells, and the organization of their bodies must of necessity appear more simple. It is, however, a deceptive simplicity; for the range of physiological activities of which the single cell is capable is no less and may indeed be greater than it is in larger animals. On the other hand, their small size enables the insects to dispense with many of the physiological elaborations that are needed by mammals. Air-containing tracheae run direct to the tissues; and the high rates of metabolism which active insects develop can be met by the diffusion of gases along these tubes, supplemented in the most active forms by mechanical ventilation of the larger trunks or of the air sacs into which they are dilated.

The needs of respiration being met in this way, the circulatory system may be correspondingly simplified. It is an open circulation, in which the body fluid or haemolymph circulates at about atmospheric pressure A valved and peristaltic dorsal vessel collects fluid from the abdomen and discharges it into the head. Accessory pumps and vessels, or pulsating membranes, ensure the subsidiary circulation in the wings, legs and antennae.

The respiratory centres in the brain and segmental ganglia, which control the ventilation of the tracheal system and the closure of its spiracular openings. are mainly influenced by the tension of gases in the tracheae and are therefore not dependent on the circulation of a nicely regulated internal medium. The elaborate behaviour of insects seems not to involve the mental processes of reasoning and

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insight; their judgement is not deranged, as Barcroft has shown ours to be, by minimal disturbances in the internal environment.

For all these reasons the insect is tolerant of much greater insults than can be borne by mammals. Some insects can survive decapitation for a year or more; many can support complete anoxia for several hours, or can be kept narcotized with pure carbon dioxide for long periods. Although temperature plays a very important part in all aspects of their physiology, most species can support a range of body temperature extending over some thirty to forty degrees centigrade— from a reversible chill coma to a reversible heat stupor.

Clearly, insects present some very desirable properties as objects for experiment. They are extremely tolerant of operation, they are so varied in form and habit that some species suited to the problem in hand can surely be found; and their small size makes it possible for the observer to be constantly aware of the whole while focusing his attention upon the part.

THE FATE OF HAEMOGLOBIN IN A BLOOD-SUCKING INSECT

As an example of the ease with which a complex series of changes may be witnessed as a whole, we may consider the fate of haemoglobin ingested by the blood-sucking bug *Rhodnius prolixus* (Wigglesworth 1943) The adult *Rhodnius* is an insect about 2 cm. in length and weighs about 80 mg. At a single meal it may ingest more than twice its own weight of blood. Most of the water in the meal is rapidly eliminated, and the concentrated blood is stored in a capacious 'stomach'. Here some part may remain undigested for a month or more, suffering no change save a partial conversion into methaemoglobin. But blood is passed on little by little to the intestine where it is broken down. Acid haematin is formed, the globin moiety is assimilated and a residue of iron porphyrin is excreted unchanged.

That represents the fate of the bulk of the blood pigment. But a small amount is absorbed without being digested, and circulates in the haemolymph. While circulating it is denatured to produce kathaemoglobin (parahaematin) which gives the body fluid a reddish tinge. Some of this kathaemoglobin is taken up by the salivary glands and converted to a deep cherry red pigment with properties resembling haemalbumen. Some is taken up by the pericardial cells—cells which are nowadays regarded as the equivalent of the reticulo-endothelial cells of vertebrates—and is there converted into biliverdin, which renders the blue-green heart as striking an object on dissection as the bright red salivary glands.

More of the pigment is taken up by the epithelial cells of the gut. Here it is converted to a brown pigment—an altered haematin which gives rise to a pyridine haemochromogen that fails to crystallize and has absorption bands at 546 and 515 mμ, as compared with 557 and 526 mμ of pyridine protohaemochromogen. Associated with this altered haematin is a green pigment of the verdohaem type, resembling the choleglobin of Lemberg, Legge & Lockwood (1939, 1941), and blue-green droplets of biliverdin. The biliverdin is finally discharged from the cells into the lumen. The iron set free in this breakdown never appears in the excreta, but

TABLE 1

arthropod species	haemolymph	salivary glands	pericardial cells	Malpighian tubes	gut wall	eggs
ANOPLURA						
Pediculus humanus	nil detectable	?	biliverdin and free iron	`	biliverdin and free iron (in lumen only)	oxyhaemoglobin
HEMIPTERA						
Cimex lectularius	haemalbumen	haemalbumen	nil	nil	nil	alkaline haematin
Rhodnius prolixus	kathaemoglobin	haemalbumen	biliverdin	nil	altered haematin, 'verdohaem', biliverdin and free iron	kathaemoglobin
Triatoma infestans	nil	nil	kathaemoglobin	biliverdin	protohaematin	nil
T. brasiliense	nil	nil	biliverdin and free iron	biliverdin and free iron (traces)	altered haematin, 'verdohaem', biliverdin and free iron	nil
Eutriatoma sordida	nil	nil	bilirubin and biliverdin	biliverdin	nil	nil
DIPTERA						
Aëdes aegypti	nil	nil	nil	nil	nil	nil
Anopheles maculipennis	nil	nil	nil	nil	nil	nil
SIPHONAPTERA						
Nosopsyllus fasciatus	nil	nil	nil	nil	nil	nil
ACARINA						
Ornithodorus moubata	alkaline haematin	—	—	nil	protohaematin	alkaline haematin
Ixodes ricinus	? methaemalbumen	—	protohaematin	nil	protohaematin	alkaline haematin

accumulates throughout the life of the insect in the cells of the gut wall, which become progressively more laden with free iron as the insect ages.

The circulating kathaemoglobin is taken up also by the follicular cells of the ovary and deposited in the yolk of the egg, so that the new-laid egg has a delicate pink tinge. This pigment suffers no further breakdown during the development of the embryo but remains unchanged in the yolk; and, since the residue of the yolk is finally enclosed in the lumen of the stomach, if the newly hatched bug is dissected, its stomach is found to contain a deep red solution of kathaemoglobin, as though it had already taken a meal of blood. The salivary glands are colourless. During the first few days of life this inherited blood pigment is digested and a black residue of haematin appears in the intestine. At the same time a little of the pigment is absorbed and taken up by the salivary glands to produce their cherry red colour; so that when the young bug takes its first meal, a little of this altered haemoglobin is reinjected into the body of its host.

If laked blood is injected into the body cavity of *Rhodnius*, all these processes are exaggerated. The salivary glands become a deeper red. The pericardial cells become loaded with the same altered haematin pigment as occurs in the gut, together with the verdohaem intermediate, biliverdin and free iron. Enormous quantities of free iron accumulate in the cells of the gut wall. The same process occurs on a small scale in the cells of the Malpighian tubes; and the excessive quantities of biliverdin displaced from the pericardial cells are largely excreted by the Malpighian tubes where they fuse in the lumen to form the most beautiful blue-green droplets. The eggs are chocolate or purplish in colour, and the changes in the young bug are correspondingly exaggerated.

The various pigments resulting from the breakdown of haemoglobin do not appear to have any physiological significance in the insect. The normal content of cytochrome and free haematin (parahaematin) in the muscles remains unchanged throughout all these vicissitudes. But the story is of interest as illustrating the freedom with which the haemoglobin molecule may be passed around the tissues, and the ease with which its fate can be followed in the insect

The extent to which haemoglobin is absorbed, and its subsequent fate, may be quite different in other blood-sucking arthropods. Some preliminary results are summarized in table 1.

WOUND HEALING IN AN INSECT

The clarity and simplicity with which a physiological reaction can be demonstrated in the tissues of the insect may be illustrated in the phenomena of wound healing (Wigglesworth 1937). The mammalian integument consists of epidermal cells, the Malpighian layer, continuously producing outer cells which in turn become progressively cornified and scale off There is therefore a continual growth for maintenance. The integument of the insect, on the other hand, is composed of an epidermis one cell thick which undergoes periods of active growth, secretes a more or less sclerotized cuticle and then becomes quiescent. There is no continuous growth of maintenance; any cell divisions that are seen after injury there-

fore represent uncomplicated growth of repair. Furthermore, since the epidermis is made up of one cell layer alone it is easy to stain and mount the integument entire and so to observe the behaviour of every epidermal cell around a given injury in a single preparation.

On this material there is no difficulty in reproducing, with a minimum of effort, the results observed on wound healing in the vertebrate—or, indeed, of extending these observations in certain particulars. Incision or excision of the epidermis results in the enlargement and 'activation' of the surrounding cells. These cells appear to be stimulated by the peptones and polypeptides or other products of protein autolysis, and leaving their attachments to the cuticle they migrate to the site of injury. If the gap is a small one they proceed to spread over it, always maintaining contact with their neighbours by means of their cytoplasmic connexions, and cell divisions rarely occur at the actual point of injury. It is in the zone around where the cells have been rendered sparse by the inward migration of their fellows that the cell divisions are most numerous   Sparseness among activated cells seems to be the stimulus to cell division.

Meanwhile there are aggregations of the phagocytic blood cells at the site of injury, but they play no essential part in the fundamental process of repair. If the gap is very small the overcrowded epidermal cells along its margins are many more than are needed for repair. When continuity is restored the excess nuclei dissolve until their normal density is regained. It is possible to produce an abnormal sparseness among the epidermal cells by blocking the anus of the insect with paraffin while it is distended by a recent meal of blood. A wound is then followed by exaggerated cell divisions. By injecting fragments of autolyzing tissue below the intact epidermis, or even applying such material to the intact surface of the cuticle, all these responses can be evoked without any interruption to the continuity of the epidermis.

Autolysis is less evident at the margin of a burn   Here the aggregation of activated epidermal cells scarcely occurs, and the repair of a burn is effected by the cells along the margins, which spread inwards dividing as they go.

The thigmotactic spreading of epidermal cells during the healing process is reminiscent of that which takes place at the margin of a colony of cells in tissue culture. It is seen very strikingly if a cylindrical fragment of a limb, for example, is implanted into the abdomen of the insect; the epidermal cells spread outwards and backwards from the cut ends until they establish continuity with each other in the form of two superimposed sleeves of epidermis—a sort of dermoid cyst (Wigglesworth 1934).

The chemotactic stimulus which initiates repair is short lived, within 3 days after making one incision the cells can be attracted away from it to a new incision close by   The stimulus that persists derives from the lack of continuity at the margins of the epithelial sheet. What remains obscure in the insect, as in the vertebrate, is the nature of this continuity, the re-establishment of which brings repair to a standstill and mediates again the 'wholeness' of the organism. But it may well be that the insect can provide material for the hopeful study of this problem—the central problem of biology (Wigglesworth 1948a).

Retention of water by the insect· the properties of the cuticle

For the most part insects are terrestrial in habit and live in comparatively dry environments. Their small size renders them particularly liable to lose water by evaporation, and the need for retaining water provides a leading motive throughout their physiology—more dominant even than it is among terrestrial vertebrates. This is evident especially in the integument of insects; and although the properties of the integument are in many ways peculiar to the group, the subject does raise problems of physiology which are not entirely devoid of general interest.

The cuticle, as we have seen, is the product of a single layer of epidermal cells. According to the needs of the case it must be rigid, flexible or elastic, and at the same time it must be waterproof. Typically three layers are recognized. an *endocuticle* of chitin-protein micelles arranged in laminae; an *exocuticle* in which, according to Pryor (1940), the protein is tanned by quinones to form a hard brown or amber coloured 'sclerotin', and an *epicuticle* which, we have recently come to realize, has a complex structure (Wigglesworth 1945*a*, 1947, 1948*b*) It seems to consist of (*a*) a basic layer, less than 1 μ thick, of 'cuticulin'—condensed lipoprotein which has probably been tanned along with the exocuticle; (*b*) a layer of crystalline waxes, which Beament (1945) estimates as having an average thickness of 0·25 μ, responsible for waterproofing, and (*c*) a layer of 'cement' protecting the wax. Cytoplasmic filaments, fifty or more from each cell, run through the cuticle and end below the wax layer.

At intervals throughout its growth the insect moults, it first lays down a new and larger cuticle and then casts off the remnants of the old. As an illustration of the varied activities of which a single cell may be capable, and of the nice timing with which these activities are co-ordinated, it is interesting to observe the stages in the deposition of the cuticle at this time.

The cells detach themselves from the old skin, multiply, arrange themselves in proper order, and then proceed to lay down the new cuticle. First they secrete the cuticulin layer of the epicuticle. This has the appearance of a somewhat refractile homogeneous sheet upon the surface; but I believe it to be pierced by cytoplasmic filaments from the cells, which will later form the pore canals. The cells next turn over to the deposition of the chitin and protein of the exocuticle. But almost at once there begins to exude from the tips of the pore canals a rather viscid fluid which will reduce ammoniacal silver. This secretion appears to consist of dihydroxyphenols, perhaps associated with protein The minute droplets enlarge and spread, and gradually unite with one another to form a more or less continuous 'polyphenol layer'. Meanwhile the secretion of protein and chitin goes on, and these layers also, destined as they are to form the exocuticle, are likewise impregnated with phenolic substances.

During this process the epidermal cells are pouring out enzymes into the fluid which separates the two cuticles—a chitinase and a proteinase presumably—which digest the chitin and protein of the old endocuticle. And the same epidermal cells absorb the products of digestion through the substance of the new cuticle.

Then, an hour or so before the old skin is shed, the inner layers of the old cuticle having been absorbed, the new cuticle is rendered waterproof by the outpouring of the wax layer. This, too, is secreted by the epidermal cells and appears to crystallize out over the surface of the polyphenol layer so that ammoniacal silver applied to the surface of the new cuticle at this time has access to the polyphenols at a few points only.

The mechanism of secretion of this wax presents some interesting problems. Chibnall & Piper (1934) suggested that these long-chain waxes might be synthesized *in situ* at the surface of the cell from more diffusible materials. Lees & Beament (1948) have recently studied the secretion in bulk of the wax with which the tick waterproofs its eggs. They suggest that the wax is solubilized by association with protein, and that the protein is detached and perhaps reabsorbed as secretion is completed.

When the old cuticle is cast the surface of the new cuticle is completely hydrophobe. Droplets of water will not leave the tip of a waxed pipette to adhere to it. But within half an hour or so after moulting the surface becomes hydrophile. That is because the numerous unicellular dermal glands which open on the surface of the cuticle now pour out their secretion of cement. This, too, appears to consist of phenol-tanned protein intimately associated with lipides. Finally, the epidermal cells secrete or activate the polyphenol oxidase and the tyrosinase which lead to the hardening and darkening of the cuticle That completes what is, I think, a rather impressive list of activities for a single cell.

All the stages in this intricate process are nicely timed and synchronized throughout the body. But almost nothing is known of the mechanism by which co-ordination is achieved. Perhaps hormones, the secretion of which is controlled by the central nervous system, are responsible. Thus Fraenkel (1935) has observed that if the blowfly (*Calliphora*), which normally becomes hard and dark within an hour of emergence from the pupa, is obliged to burrow continuously through the soil, it will remain soft and pale for 6 or 7 hr. and then proceed to darken when it becomes free. Something of the same sort is seen in the mosquito larva *Aëdes* (Wigglesworth 1938b). When this larva hatches from the egg the tracheal system is full of liquid. The young larva rises to the surface of the water, opens its spiracles to the atmosphere, and after a pause the liquid is slowly absorbed through the tracheal walls and the system fills with air. Absorption may be delayed for 3 days by keeping the larva submerged and yet take place normally on exposure to the air; but after 4 days some change has supervened and filling is never complete. Likewise, if the larvae of fleas are allowed to hatch in water and kept submerged for several days the tracheal system will no longer fill with air when the larvae are dried (Sikes & Wigglesworth 1931) If the newly hatched mosquito larva is narcotized with chloroform and then placed with its spiracles open to the air, filling does not take place. After recovery from the anaesthetic there is still a pause of 10 min or so and then the process begins. Clearly the nervous system is involved; yet there is no reason to suppose that the cells bounding the tracheal system are supplied with nerves.

Recent work on the cuticle has emphasized the constant association of chitin and protein. Indeed, in the latest classification of mucoproteins and the like

(Stacey 1943; Haworth 1946), 'chitin' in the loose sense is referred to the group of mucopolysaccharides. That recalls the fact that, so far as is known, insects do not secrete mucus. In those forms which ingest with their food solid particles that might be expected to injure the delicate epithelium or which, maybe, are derived from ancestors that fed upon such things, the contents of the gut are separated from the wall by a so-called peritrophic membrane. This consists of chitin and protein, closely bound together. In many insects, such as caterpillars, grasshoppers and bees, it is produced as a secretion by the cells of the mid-intestine throughout its length and condenses as a many-layered sheath around the food. In other insects, of which the earwig, muscid flies, or the larvae of mosquitoes are examples, it is produced as a viscid secretion by a special group of cells at the anterior end of the mid-gut. This secretion is passed through an annular press in which it is condensed and moulded to form a cylindrical tube of uniform diameter and consistency in which the gut contents are enclosed. These annular moulds are of varied form and often of very elegant design (Wigglesworth 1929, 1930a, 1939).

THE ROLE OF WATER IN EXCRETION

Throughout the animal kingdom water is a dominant factor in the excretion of waste nitrogen. The majority of insects, like birds and reptiles, eliminate their nitrogen almost wholly in the form of uric acid. The characteristic organs of excretion are the Malpighian tubes, blind vessels which lie free and independent of one another in the body fluid and discharge into the lumen of the gut where the proctodoeum joins the mesenteron. The Malpighian tubes afford exceptional opportunities for studying the physiology of excretion. In place of the tangled mass of tubules which compose the kidney, the physiologist can observe a single unravelled vessel in which it is easy to see in the living state any histological changes that take place.

In *Rhodnius* there are four Malpighian tubes, some 4 cm. in length, each of which consists of an upper and a lower segment (Wigglesworth 1931a). Both segments are lined by a conspicuous striated border the characters of which, as seen in histological sections, appear to be identical throughout. There has been much controversy in the past as to whether the striated border which lines the kidney tubules, for example, is really a 'brush border' made up of independent filaments arising from the cells or a 'honeycomb border' composed of rods or vesicles organically united with their neighbours.

It is interesting to observe that in the Malpighian tubes of *Rhodnius*, examined in the fresh state in the body fluid of the insect itself (very misleading results are obtained if so-called 'physiological solutions' are employed), the upper segment has a honeycomb border and the lower segment a brush border. There is no gradual transition from one type to the other, the transformation is complete in adjacent cells. The lumen of the upper segment contains a clear fluid; the lower segment is filled with crystalline spheres of uric acid, and the smallest spheres can often be seen to lie in the free space between the filaments. Furthermore, during the early stages of excretion following a meal of blood, these filaments show some remarkable

changes. The uric acid granules are first washed out as the excess fluid is eliminated. Then the filaments grow out and extend across the lumen, where they can be seen waving passively to and fro Later the entire lumen may be filled for a time with these intermingling filaments entangling the granules of uric acid which are held stationary as in a gel. Later still the filaments contract down and the crystalline spheres are left free in the lumen. The interesting point is that I have never been able to recognize these curious changes in fixed material.

Solid uric acid appears in these tubes almost immediately below the change in cell type. The evidence goes to show that uric acid or urate is secreted in solution by the upper segment, while water and perhaps base are reabsorbed in the lower segment, leading to the crystallization of the free acid. For diffusible dyes, such as neutral red, introduced into the body fluid are quickly taken up by the cells of the upper segment, are discharged into the lumen, and only then appear in the cells of the lower segment. Indicators such as phenol red likewise appear first in the lumen of the upper segment; here the contents are faintly alkaline (pH 7·2). In the lower segment the contents are definitely acid (pH 6·6). Finally, it is possible to apply ligatures to the Malpighian tubes, in the form of tiny wax clamps, at a time when the uratic contents have been washed out. If a length of the lower segment is isolated in this way it shows no distension and no uric acid is deposited within it. Uric acid accumulates above and below the ligated section. (That below has clearly come from the other tubes and entered from the proximal extremity.)

One must conclude that there is a continuous circulation of water and perhaps of base carrying uric acid from the system and then being reabsorbed. After ingesting a feed of blood, *Rhodnius* excretes within the first 3 hr some 75% of the water in its meal. It may subsist on the remainder for 6 weeks or more, eliminating uric acid, during the first 2 weeks at least, at an average rate exceeding 0·5 mg /day—the weight of the insect being about 80 mg.

Not all insects have such distinct secretory and reabsorptive segments in the Malpighian tubes In some, such as the mosquito *Aedes*, uric acid appears in solid form in the lumen throughout the length of the tubes. Perhaps the same cells are concerned in both secretion and reabsorption That is not known. But it is certain that insects are capable of holding uric acid in solution at concentrations far above saturation. Many insects deposit uric acid in solid form in various cells in the epidermis or in the fat body. In the larva of *Aedes* (Wigglesworth 1942a), alongside the reserve deposits of glycogen and fat, the cells of the fat body contain watery vacuoles. In the starved insect the reserves slowly disappear and the cells are filled with these greyish watery vacuoles alone, in a few of which a tiny crystal of uric acid can sometimes be seen. It is easy to observe a selected living cell from day to day under the highest powers of the microscope and then to add a fixative and to see that in each vacuole large wheatsheaf crystals of uric acid separate out.

In other insects, such as the louse *Pediculus* or the mealworm *Tenebrio*, the Malpighian tubes contain a clear fluid throughout their length. Uric acid separates only in the rectum, where there are large epithelial cells sometimes aggregated into so-called 'rectal glands'. There can be little doubt that these cells are concerned

in reabsorbing water (Wigglesworth 1932). In the mealworm, which is particularly well adapted to conserve water, the mixed urine and faecal residue is reduced to a bone-dry powder before it is discharged.

The rectum of the mosquito larva also has a lining of large epithelial cells. These too are clearly absorbing water; for it can readily be seen in the living insect that less water is being discharged from the anus than is reaching the rectum from the Malpighian tubes (Wigglesworth 1933b). The aquatic larva of the mosquito is, of course, under no necessity to conserve water. This reabsorption is probably for the sake of valuable solutes such as chlorides (Boné & Koch 1942). It may well be that the rectal glands of terrestrial insects are equally concerned in the conservation of chlorides and other salts (Patton & Craig 1939).

In addition to these enlarged rectal cells, mosquito larvae have conspicuous 'anal papillae' (sometimes referred to as 'anal gills'—although they have almost no respiratory function (Wigglesworth 1933b)), which are hollow finger-like protrusions made up of large epidermal cells. These are covered by a very thin cuticle which is readily permeable to water and to salts. Indeed, their epidermal cells provide wonderful material for observing the effects of ions on the cell substance—the swelling and dispersive action of monovalent ions, the cohesive action of divalent and trivalent ions (Wigglesworth 1933a). Water is continually entering through these papillae to be excreted by the Malpighian tubes, but their main function seems to be the active uptake of chloride from the medium (Koch 1938). A larva, whose blood chloride has been reduced from 0·3 % NaCl to 0·05 % by keeping for some days in distilled water, can restore its chloride content to the normal level within 24 hr. if placed in tap water with a chloride content of only 0·006 %. This it cannot do if it is deprived of its anal papillae. If larvae are reared in distilled water containing minimal quantities of chloride they show a functional hypertrophy of the anal papillae (Wigglesworth 1938a). Surely there is material here well suited for the study of some of the more subtle problems of secretory activity.

WATER RETENTION IN RESPIRATION

All terrestrial animals are obliged to make some compromise between the need for holding water and the need for obtaining oxygen. In insects this is achieved by the provision of sphincters at the spiracular openings of the tracheal system. These are held firmly closed most of the time and opened only enough to meet the oxygen requirements (Hazelhoff 1926). In most insects, indeed, the greater part of the water loss takes place by evaporation through the spiracles, and if these are kept open by exposure to 2 % carbon dioxide the insect rapidly dries up (Mellanby 1934, Wigglesworth & Gillett 1936).

The regulation of these respiratory movements, the opening and closing of the spiracles, presents some interesting parallels and contrasts with the control of respiratory movements in mammals. The matter can be very conveniently studied in the flea, which is a small insect that can readily be enclosed in a gas chamber and the opening and closing of the spiracles observed by transmitted light under the microscope (Wigglesworth 1935). There are two pairs of thoracic and eight

pairs of abdominal spiracles* of which the thoracic and the 1st and 8th abdominal are the most important in respiration. These spiracles are held closed in the fasting flea at rest. They open within a few seconds after muscular movements commence. When the general rate of metabolism is increased by feeding or by high temperature, they show a regular rhythmic opening and closing every few seconds. During the height of digestion, or during the development of eggs in the female, they are held continuously open.

Both oxygen lack and carbon dioxide excess contribute to the control of the respiratory rhythm. The closed spiracle is caused to open mainly by the exhaustion of oxygen in the tracheal system; but the amount of carbon dioxide which has accumulated in the tissues during the period of closure determines the length of time the spiracle will remain open. The duration of the open period is therefore more or less proportional to the duration of the closed period that precedes it. If the closed period is very short, as it is at high temperatures where the intensity of metabolism is increased or at low oxygen tensions where the oxygen in the system is soon used up, the open period is likewise shortened. Whereas at low temperatures or in pure oxygen, where the closed period is greatly prolonged, the open period is also prolonged.

The solubility of carbon dioxide in the tissue fluids has, indeed, the same effect in slowing up the respiratory responses as is characteristic of this gas as a respiratory stimulant in mammals. But the immediate stimulus to the respiratory centres, causing the spiracles to open, is probably acidity. Traces of lactic acid introduced into the circulation of the flea will shorten the respiratory rhythm, larger amounts will cause the spiracles to remain permanently open; and the same results appear if the flea is enclosed in a bubble surrounded by alkaline pyrogallol, in which both oxygen and carbon dioxide will be removed from the tissues.

The respiratory centres controlling these movements are distributed throughout the ganglia of the nervous system. Removal of the brain has no effect. After removal of the thoracic ganglia the response in the abdomen remains, although the sensitivity is reduced, and there is a very slow reaction persisting, the nervous basis of which is not known, even when the entire central nervous system of the flea has been eliminated.

Thus the flea shows several adaptations to diminished concentrations of oxygen. Oxygen want is more quickly felt when the spiracles are closed; hence the centres are stimulated sooner and the spiracles open more frequently. There is an incomplete oxidation of metabolites, which probably lowers the pH towards the threshold level of stimulation of the centres and in this way contributes to the more frequent opening of the spiracles. The accumulation of metabolites indicates that the oxygen tension in the tissues is reduced. By increasing the difference in partial pressures, this will increase the rapidity of diffusion from the spiracle.

There is another interesting phenomenon which results from the incomplete oxidation of metabolites (Wigglesworth 1930b, 1931b, 1935, 1938a). The tracheae

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* In the paper quoted the author followed Lass (1905) in regarding the first abdominal spiracle as the third thoracic. It is more likely that the flea, like other insects, has only two pairs of thoracic spiracles.

end by breaking up into very fine thin-walled tracheoles. The lining membrane of these is permeable to water, which can thus enter the lumen and be drawn along them by capillarity. The height to which this fluid rises is determined by the balance between the capillarity of the tracheole, which diminishes, of course, as the diameter increases, and the swelling pressure of the cytoplasm bounding the wall of the vessels. The swelling pressure may be influenced by changes in the surrounding tissues or tissue fluids, notably by the changes in osmotic pressure which result from the incomplete oxidation of metabolites, so that in the asphyxiated insect the liquid is absorbed and air extends into the fine endings of the tracheoles. This has the effect of increasing the surface area over which oxygen can diffuse into the tissues and of doing this most rapidly in those tissues which are most active and whose needs are therefore greatest.

In the abdomen of the flea at rest in air the liquid in the tracheoles rises high up the tubes. After violent muscular activity at room temperature, the air extends a little farther down. At 35° C this downward movement of air after struggling is more extensive; indeed, in many of the tracheoles the meniscus is moving up or down almost the whole time. But in the insect at rest there is no difference in the equilibrium level at 17 or 35° C. On the other hand, when the oxygen in the air is reduced to say 10%, the equilibrium position in the tracheoles when the flea is at rest is definitely changed; the air extends more deeply into the tissues. In a 5% mixture of oxygen the air extends still further, and in nitrogen containing 0·8% of oxygen the gas reaches such fine tubes that its limit cannot be seen with the microscope.

Clearly there is an accumulation of metabolites that is arrested at a point which depends upon the partial pressure of oxygen in the atmosphere. Equilibrium between oxygen supply and oxygen consumption is then restored, a constant level of oxygen debt remaining. Even in air a certain accumulation exists, which can be removed in oxygen; for on going from air to oxygen the equilibrium point to which gas extends in the tracheoles, retreats appreciably towards the wider part of the tubes.

HORMONES CONTROLLING GROWTH AND REPRODUCTION

Since the days of Brown-Séquard it has been known that growth in vertebrates is largely controlled by circulating hormones coming in part from cells of internal secretion associated with the gonads, in part from special ductless glands. Attempts were early made to repeat these observations on insects. But neither castration nor implantation of gonads had any influence upon their secondary sexual characters, and, quite gratuitously, the general conclusion was drawn, and the dogma became accepted, that insects have no endocrine organs and secrete no hormones.

In recent years this conclusion has been disproved, and the regulation of growth by chemical means has been demonstrated in many insects. The possibilities of the material may be illustrated from the observations made on *Rhodnius*, which has proved admirably suited to studies of this kind (Wigglesworth 1934, 1936, 1940a, 1940b). *Rhodnius* has five larval and nymphal stages before it turns into an adult; and in each of these it takes a single gigantic meal of blood. The nymphs

have great powers of resistance to starvation and can survive unfed for many weeks. But if they get a meal of blood above a certain size, which distends the abdomen above a certain degree, a nervous stimulus is carried to the brain and causes certain large nerve cells (neuro-secretory cells), lying in the dorsum of the protocerebrum, to secrete a 'moulting hormone'.

Under the influence of this hormone, the epidermal cells leave the old cuticle, grow and divide. Mitosis is, indeed, so exuberant that far more cells are produced than will be needed to lay down the new cuticle (Wigglesworth 1942b). The unwanted cells therefore die. Even while still more cells are being produced, scattered everywhere among them are basophil droplets derived from the dissolution of the unwanted nuclei. This fever of growth and decay lasts for several days, but by 10 days after feeding it is almost at an end; the excess nuclei have melted away, and a regular epithelium with nuclei regularly spaced is ready to begin the deposition of the new cuticle. In due time the old skin is cast off; at a fixed interval after feeding the insect moults.

Throughout the five nymphal stages there is comparatively little change in the body form of *Rhodnius*. The abdomen, for example, bears scattered plaques each carrying a bristle between which the epicuticle is thrown into star-shaped folds. In order to accommodate the great meal of blood that is taken in each instar the inner layers of the cuticle stretch, the stellate folds become partially smoothed out. In the adult, on the other hand, bristles are very scanty on the upper surface of the abdomen, there are no plaques, the epicuticle is thrown into folds running transversely across the abdomen, and the outer parts of the cuticle are sclerotized and therefore incapable of being stretched. The distending meals of blood are accommodated in the adult by the provision of a pleat along either side of the abdomen. This first unfolds, and then a part of it, composed of soft elastic cuticle, stretches to a prodigious extent (Wigglesworth 1933c).

These detailed differences in the cuticle of the abdomen provide a useful indication of the degree of metamorphosis achieved when the abdominal cuticle is viewed by itself. But the more closely the general changes that take place at the moulting of the 5th stage nymph are studied, the more striking does the metamorphosis to the adult appear. There are elaborate changes in the thorax with the development of wings: climbing or adhesive organs appear on the tibiae of the first two pairs of legs (Gillett & Wigglesworth 1932); ocelli are formed for the first time; the colour pattern of the abdomen is quite different; and the complicated external genitalia are developed.

If a *Rhodnius* nymph is decapitated within a day or so after feeding, it is deprived of the source of the moulting hormone in the secreting cells of the brain, and it fails to moult—although such headless insects have remained alive for more than a year. Some 4 to 8 days after feeding there is a critical period which marks the time when sufficient of the moulting hormone has been secreted, and when this period has passed moulting takes place even in the decapitated insect. If the nymph decapitated soon after feeding is joined by means of a capillary tube to a nymph with the head intact or to a nymph that has passed the critical period, so that the blood flows from one to the other, the decapitated nymph is caused

to moult. Moulting can likewise be induced in the headless nymph by implanting into its abdomen that region of the brain which contains the neuro-secretory cells.

It is characteristic of many insects that from time to time growth ceases and they enter upon a state of 'diapause'. The arrested growth which supervenes in the decapitated *Rhodnius* nymph has much in common with natural diapause; which suggests that the immediate cause of diapause may be a failure in the secretion of the hormones necessary for growth. This idea has been substantiated by the recent work of Williams (1946, 1947, 1948) on the pupal diapause of the giant silk moths. Doubtless many different factors may be responsible in different insects for the arrest of secretion. In *Rhodnius* a natural diapause does not occur—save in those insects which do not ingest a meal large enough to stretch the abdomen to the requisite degree. But the normal *Rhodnius* always harbours in its gut a symbiotic bacterium, *Actinomyces rhodnii*, which is essential for the nutrition of its host (Brecher & Wigglesworth 1944). Apparently it produces certain vitamins, probably of the B group, which are deficient in blood. If *Rhodnius* is reared under sterile conditions so as to be freed of its *Actinomyces*, it goes into a state of diapause and will not grow beyond the 4th or 5th stage. Perhaps the vitamins synthesized by this micro-organism provide the raw material for the production of the moulting hormone.

This moulting hormone secreted by the brain appears to be the same in each nymphal stage. But the characters developed by the moulting insect, that is, the occurrence or non-occurrence of metamorphosis, are determined by another hormone (or group of hormones) secreted by the corpus allatum. The corpus allatum bears much the same relation to the neuro-secretory cells of the proto-cerebrum as the hypophysis of vertebrates does to the neuro-secretory cells of the hypothalamus—it is a small ductless gland which appears to derive its nerve supply from that source (Hanström 1941; Scharrer & Scharrer 1944). Throughout the early nymphal stages the corpus allatum secretes what may be called a 'juvenile hormone', the function of which is to maintain the youthful character of the insect and to deter it from metamorphosis into an adult before it is fully grown. During the moulting of the first four nymphal stages this secretion is active and nymphal characters are retained; during the moulting of the 5th stage nymph the corpus allatum no longer secretes the juvenile hormone, the latent adult characters are realized and metamorphosis takes place.

Recently some evidence has been obtained that the corpus allatum of the 5th stage nymph not only ceases to secrete the juvenile hormone but actively absorbs from the blood any traces of the hormone which remain (Wigglesworth 1948c). If that is confirmed it will represent a new principle in the activity of endocrine organs. Later still, in the adult insect, the corpus allatum begins once more to secrete the juvenile hormone, and this secretion is necessary for the deposition of yolk in the eggs by the female and the full development of the accessory sexual glands of the male.

Thus if any insect of the first four nymphal stages is induced to moult by transfusion from the moulting 5th stage nymph it will suffer a premature metamor-

phosis. Even the 1st stage nymph, transfused from the moulting 5th stage insect, will turn into a diminutive adult. Conversely, if corpora allata from 3rd or 4th stage nymphs are implanted into the abdomen of the 5th stage nymph, when this moults it develops nymphal characters again, instead of transforming into an adult. The experiment may be repeated, and a giant 7th stage nymph has been produced. Sometimes the experiment is less successful and, instead of an extra nymphal stage being produced, an adult is developed which has a little patch of nymphal cuticle over the site at which the corpus allatum was implanted. Around such a patch the cuticle is intermediate in character, and gradually this merges into cuticle of adult type.

One must regard the growing insect as a differentiated continuum or mosaic in which there exist two systems, or one system susceptible to two types of development, nymphal and adult. Reaction with the appropriate hormones decides when and which of these shall become manifest. Taking the simplest case of an ordinary epidermal cell of the abdominal wall, one may picture the cell as containing two enzyme systems. One produces adult cuticle and is activated by the moulting hormone. The other produces nymphal cuticle and is activated by the moulting hormone only in the presence of the juvenile hormone—which may perhaps be looked upon as a sort of 'coenzyme'. In the presence of this coenzyme the nymphal system takes precedence over the adult, and nymphal cuticle is laid down.

It is interesting to note that even after metamorphosis to the adult form, the nymphal system may persist latent within the cells. For it is possible, by transfusion with blood from several moulting 5th stage nymphs, to induce the adult *Rhodnius* to moult again and to develop a new cuticle whose characters are wholly adult. But if a number of corpora allata from 3rd or 4th stage nymphs are first implanted into the abdomen, so that juvenile hormone is present in the blood, there is a partial *reversal* of metamorphosis when the adult moults; the cuticle of the abdomen develops plaques and folds approximating to those of the nymph. It shows, in fact, a genuine rejuvenation.

In the converse experiment, when metamorphosis is produced experimentally in the 1st stage nymph soon after hatching from the egg, the epidermis of the miniature adult that results is made up of less than one-hundredth part of the number of cells which compose the normal adult. This simple observation alone shows how limited is the importance of the cells in defining the form of the body. The cells do not 'co-operate to mould the body form', they seem merely to carry and care for a small segment of the continuum which is the organism and of which they are the servants. The movements and secretions which bring about the visible process of growth and metamorphosis are, of course, the products of the cells. But the cells are only the agents of the pervading web which is the organism itself (Wigglesworth 1945b, 1948a).

Whether insects will provide good material for the isolation and characterization of hormones by chemical means remains to be seen. But they can unquestionably afford wonderful material for the study of those subtle problems connected with the analysis of growth and its regulation, with the moulding of the body form, and ultimately with the nature of the organism.

CONCLUSION

It has not been possible in the course of one lecture to give a systematic account of what is peculiar in the physiology of insects. It seemed to me, moreover, that it would serve no useful purpose to devote our time wholly to generalities. I have therefore chosen a few specific examples, drawn for the most part from those aspects of the subject with which I happen personally to have been concerned, and have submitted these in the hope of exciting the sympathetic interest of the physiologist. It is scarcely needful to point out that the studies which have been made are of necessity superficial, and the account of them presented in this lecture more trivial still. This is a world in which nuggets in profusion lie upon the surface. But I hope I may have said enough to prove that rich veins of gold await the real specialist who cares to utilize the insect as a medium for the advancement of physiology.

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On the time required for diffusion and its relation to processes in muscle

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Two problems of diffusion are considered in relation to muscle

(A) How rapidly can chemical changes occurring inside a muscle be detected and measured outside by some device (e.g. an electrode) specifically sensitive to one of the products capable of diffusing out?

(B) If, as seems probable, the process of excitation is limited to the surface of a fibre, could diffusion be rapid enough for contraction to be propagated inwards by the arrival at each point of some chemical substance liberated at the surface?

As regards (A), it is shown that an indication of chemical change can be given rapidly if the layers of tissue and fluid between muscle fibre and recording surface are kept thin, but that the attainment of final equilibrium is too slow to allow accurate following of changes occurring at all quickly, unless the layers of tissue and fluid are reduced to a few microns in thickness.

As regards (B), it is shown that if the twitch of a muscle fibre is assumed to involve the contraction of the whole of its contents, diffusion from the outer surface could not be fast enough to account for observed speeds of contraction. If, however, in a twitch the outer region only, say one-half of the fibre, were involved, which agrees with the known fact that the ratio of twitch tension to tetanus tension is usually considerably less than unity, then diffusion over the shorter distances involved would be amply quick enough. The considerable difference between the temperature coefficients of the rates of diffusion and contraction shows that the time course of contraction cannot be determined by diffusion. The times calculated for diffusion, however, are short enough to allow us to suppose, if we wish, that the contractile process begins at any point when the concentration there of some hypothetical substance liberated at the surface during excitation, and diffusing inwards, reaches a critical value

The connexion between excitation and contraction is discussed.

In an earlier paper (Hill 1928) problems of diffusion in living tissues were discussed, particularly in relation to respiration in muscle and nerve. In the present paper two special questions are considered. (A) How rapidly can chemical changes

occurring inside a muscle during contraction be detected and measured outside by an electrode or similar device specifically sensitive to one of the products capable of diffusing outwards? (B) If the process of excitation is limited, as it probably is, to the surface of a muscle fibre, how rapidly could a twitch develop if its onset at any point depended on the arrival by diffusion of some chemical substance (e g. calcium ions or acetyl choline) liberated at the surface during excitation?

(A) Ordinary biochemical methods of investigating chemical change in living muscle are very slow and insensitive compared with various physical methods, and require the destruction of the tissue each time an estimation is made Even at 0° C the cycle of contraction and relaxation in the twitch of a frog's striated muscle is over in 1 sec., so that the accurate resolution of chemical events into their several phases would require measurement of a few micrograms of material within small fractions of a second. The difficulty cannot be avoided by applying a prolonged stimulus instead of a single shock, so obtaining longer times and more material for estimation, for that merely runs the processes of contraction, relaxation and recovery set up by successive elements of the stimulus into one another and gives a blurred picture of the result The difficulty has led biochemists to the study of enzyme systems *in vitro* in which the requirements of time and quantity are less rigorous. Suggestive, however, as the results of such studies are, they cannot inform us directly about the actual processes in living muscle, and other methods are required.

The consumption of oxygen and the liberation of carbon dioxide by muscle can be followed continuously, though with considerable time-lag, and alterations of pH can be inferred from changes in the release of carbon dioxide, or measured directly by an electrode in contact with the surface Such methods depend on diffusion in and out, not on the destruction and chemical analysis of the tissue; their speed is determined by the rate of attainment of a steady state by diffusion and is greater the less the amount of material required to be transferred. The ideal is to bring a recording surface specifically sensitive to one of the products of chemical reaction into the closest possible contact with the outside of the tissue and to follow there the changes occurring inside A glass electrode for measuring pH (Dubuisson 1939) is a particular application of the principle, but others are possible. The object of the present discussion is to define the limits set by diffusion to the speed at which such a system can operate.

The problem can be stated as follows. A plane sheet of muscle (which in the times considered can be regarded as of infinite thickness) is separated from a recording surface by a layer b cm. thick of inert tissue and fluid Let y_0 be the sudden change, at time zero, of the concentration inside the muscle of the chemical substance considered, y the resulting change of concentration at the recording surface at time t. Then, if erf (a) is the probability integral $\dfrac{2}{\sqrt{\pi}} \displaystyle\int_0^a e^{-x^2} dx$,

$$y/y_0 = 1 - \mathrm{erf}(b/2\sqrt{(kt)}). \tag{1}$$

The relation between y/y_0 and kt/b^2 is shown in figure 1. For quantitative discussion we need to know k and b. The diffusion constant through living material

*cannot be greater than through water; it may be much less if membranes intervene.
In water the temperature coefficient of the rate of diffusion of most substances
is about 1·33 per 10°; calculated for 0° C, with seconds as units of time, the values
of k are roughly as follows for various substances:*

Cane sugar, $2·3 \times 10^{-6}$; glycerol, $3·4 \times 10^{-6}$; oxygen, $4·2 \times 10^{-6}$; carbon dioxide,
$5·0 \times 10^{-6}$, urea, $6·0 \times 10^{-6}$; $CaCl_2$, $6·0 \times 10^{-6}$; ethyl alcohol, $6·1 \times 10^{-6}$; acetic
acid, $6·1 \times 10^{-6}$, KCl, $9·6 \times 10^{-6}$.

These values were calculated from Landolt & Börnstein's *Tables* (1912) for diffusion
through water at 0° C, except for oxygen and carbon dioxide which are diffusion constants
through living tissue calculated (Hill 1928) for 0° C from Krogh's observations.
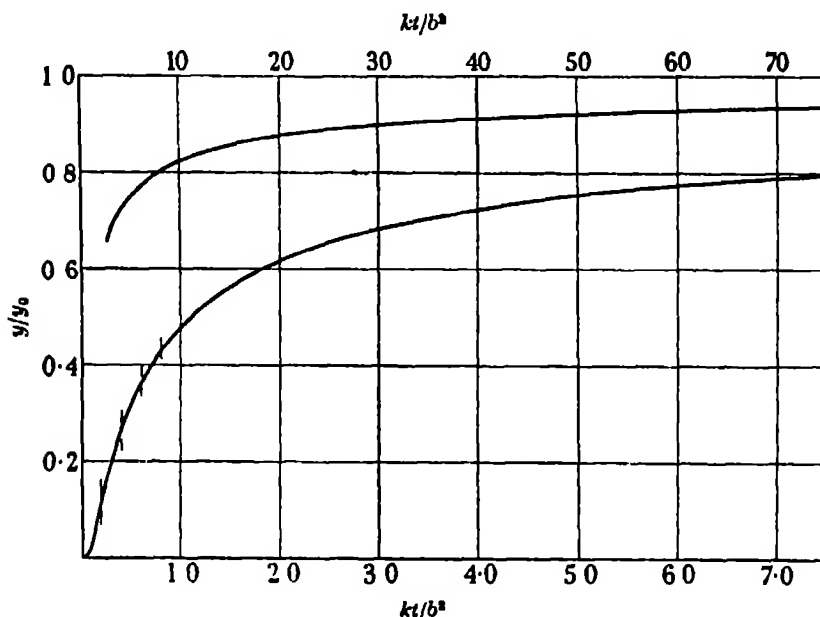
FIGURE 1. Calculated relation (equation (1)) between y/y_0 and kt/b^2 The two curves are on
different scales of kt/b^2, shown below and above.

If we consider specifically the case of a glass electrode we may assume (Dubuisson
1939) that a change of pH inside a muscle is transmitted outside by the diffusion
of carbon dioxide or other weak acid, so that if the boundary of the fibres provides
no barrier k, at 0° C, may be taken as 5×10^{-6}. Whatever substance we con-
sidered k could not be much more, it might be much less. The calculation, therefore,
is optimistic.

As regards b, muscles on the surface of the body are covered, on the side towards
the skin, with a sheet of connective tissue which in the frog is several microns,
3 to 7 μ, thick. To this must be added the layer or film of fluid between the tissue
and the recording surface, making (say) $10 \mu = 10^{-3}$ cm. in all.

With these values of k and b, $k/b^2 = 5$, so that figure 1 gives the concentration
at the recording surface at times equal to one-fifth of the values of kt/b^2 along the

axis. The result shows that *an indication* outside of a change inside is given very quickly. For example, 20 % of the final value is reached at $kt/b^2 = 0.31$, $t = 0.062$ sec., which at 0° C is only about one-fifth the duration of the contraction phase of a single twitch; while half the full value is reached at $kt/b^2 = 1.1$, $t = 0.22$ sec. The final value, however, is attained only very slowly, even 90 % of it requires $kt/b^2 = 31$, $t = 6.2$ sec., six times the duration of a twitch. Thus with a rapidly changing concentration inside, accurate measurement would be impossible outside because of the slowness of the later stages of diffusion; the details of the picture would be rounded off and obscured, just like the record of a rapidly changing current made with a heavily overdamped galvanometer.

If b could be reduced, i.e. if closer contact could be made between muscle and recording surface, the speed might be largely increased. If, for example, b were 5μ instead of 10μ, k/b^2 would be 20 instead of 5 and the times would be reduced to one-quarter. All connective tissue, therefore, should be avoided and a minimum of fluid allowed. Dubuisson (1939, p. 464) with his glass electrode was aware of the extreme importance of a very thin layer of liquid between muscle and electrode. The avoidance of connective tissue is equally important. The inner surfaces of muscles such as the frog's sartorius have very little of it and are preferable to the outer, the gastrocnemius with its thick perimysium should not be used.

These calculations refer to a muscle at 0° C, and it might be thought that a higher temperature would make things easier because of the greater speed of diffusion. In fact, however, it would make them more difficult. The rate at which chemical events occur in a muscle twitch, as shown by the rate of heat production, is more than doubled by a rise of 10° C, and the speed of diffusion increases by only one-third. The highest resolution, therefore, is obtained by working at the lowest possible temperature, a three-fold advantage is gained by changing from 20 to 0° C.

We have assumed hitherto that no delay occurs at the recording surface itself. According, however, to Dubuisson (1939, p. 465) his glass electrode required about 4 sec. for an instantaneous change in pH under the electrode to be translated into a steady final deflexion. The delay cannot be due to the time constant (resistance × capacity) of the glass membrane which should be at most 50 to 100 msec. Judging from the calculations given above it may very well be due to time taken in diffusion in the glass (10 to 20μ thick). The glass membrane, therefore, should be of the utmost thinness, and (as we have seen above) as close as possible to the muscle fibres.

(B) The second problem can be stated as follows. Suppose that some chemical substance X is liberated suddenly at time $t = 0$ all over the surface of a cylinder of radius a, and proceeds to diffuse inwards (but not outwards) with diffusion constant k. Let y be its concentration at time t at a point distant r from the axis, y_∞ its final concentration after diffusion is complete. We assume that X is not used up during its passage across the cylinder, that any reactions it undergoes occur later. The treatment is similar to that given by Hill (1928, p. 70)* and the solution,

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* On p. 71 of that paper an error occurs in equations (45) and (46), the -2 before the square brackets should read $+2$. The curves were calculated correctly.

for which I am greatly indebted to Mr L. A. Wigglesworth, of University College, London, is

$$\frac{y}{y_\infty} = 1 + \sum_{\alpha_1, \alpha_2, \dots} \frac{J_0(\alpha r/a)}{J_0(\alpha)} e^{-\alpha^2 kt/a^2}, \tag{2}$$

where α_1, α_2, α_3, ... are the positive zeros of $J_1(\alpha)$. A deduction of this equation is given in the appendix.
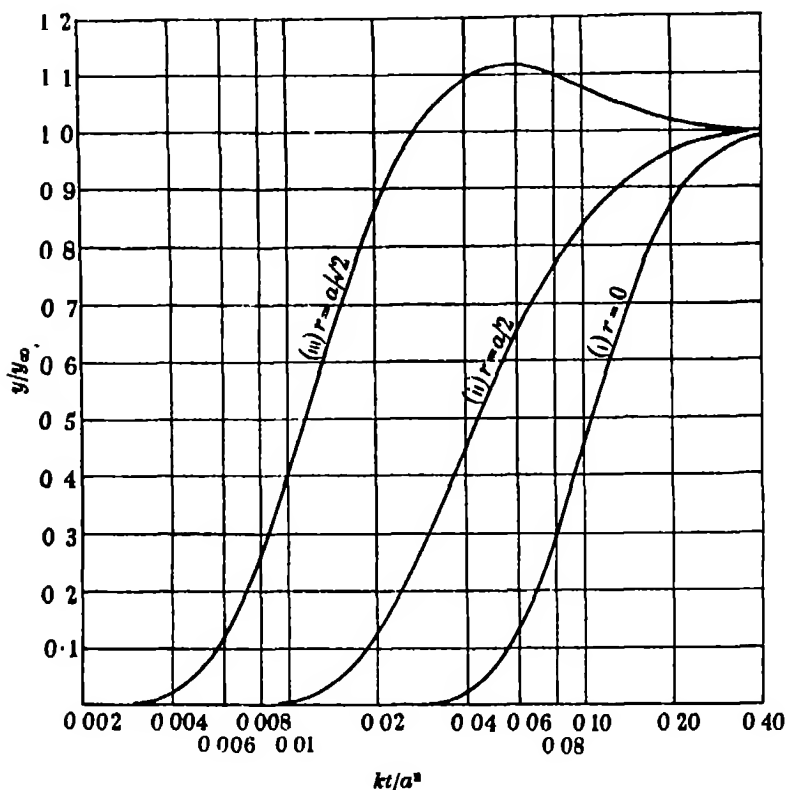
FIGURE 2. Calculated relation (equation (2)) between y/y_∞ and kt/a^2. kt/a^2 is given on a logarithmic scale. Curves (i), (ii) and (iii) are respectively for points on the axis and for points distant $0.5a$ and $0.707a$ from the axis.

The calculated relation between y/y_∞ and kt/a^2 is shown in figure 2, kt/a^2 being given on a logarithmic scale, for the following cases. (i) $r = 0$, (ii) $r = a/2$, (iii) $r = a/\sqrt{2}$. For (i), the diffusing substance would have to cross the whole volume of the cylinder to reach the region considered, for (ii) and (iii) respectively three-quarters and one-half the volume. The distances traversed from the surface inwards, in the three cases, are in the ratios $1 : 0.5 : 0.293$, and the ratios of the times at which any specified value of y/y_∞ is reached are about as the squares of these numbers. Curve (iii) is interesting in rising above its final steady value before settling down. This is intelligible when we recall the initial assumption

that a 'pulse' of X is liberated at time zero at the surface; this 'pulse' would produce locally a high concentration which would be propagated inwards a certain distance before being smoothed out.

Consider a muscle fibre of 100μ diameter ($a = 0.005$ cm.) in the frog's sartorius at $0°$ C. Taking $k = 5 \times 10^{-6}$ as above, $kt/a^2 = 0.2t$, and the times for a given degree of diffusion are five-fold the values of kt/a^2 shown in figure 2. The first question to be asked is: *Could diffusion work fast enough to affect the whole volume of the fibre in the available time of a single twitch?* The contraction phase of a twitch at $0°$ C is complete in 0.3 to 0.5 sec. If we allow 0.2 sec. as the time available for diffusion, $kt/a^2 = 0.04$ and from curve (i) $y/y_\infty = 0.02$. Unless, therefore, X were produced in large excess it would not arrive in time to induce contraction on the axis of the fibre. This conclusion is strengthened if we consider (1) large fibres, or (2) higher temperatures. For a 150μ fibre, at 0.2 sec. $kt/a^2 = 0.018$, so y/y_∞ at 0.2 sec. is effectively zero. At a higher temperature diffusion is more rapid, but so is contraction. At $30°$ C k might become 1.2×10^{-5}, but the available time in a twitch would be reduced to about 0.02 sec. For a 100μ fibre, kt/a^2 would then be about 0.01 and y/y_∞ on the axis would be zero. Even in a 50μ fibre, which is below the mean size, kt/a^2 would be 0.04 and again y/y_∞ would be practically zero.

Consider a very different muscle, the internal rectus of the eye of a kitten (Denny-Brown 1929, figure 4 (*f*), (*g*) and (*h*), p 392, and plate 22, figure 18). The diameter of the fibres was about 25μ and the 'twitch duration' (mechanical onset to 'angle') about 8.5 msec , the available time for diffusion therefore could be taken as 5 msec. At $37°$ C k might be 1.4×10^{-5}. From these figures $kt/a^2 = 0.045$ for which y/y_∞ on the axis is 0.04. The same conclusion is reached as in the case of the frog's sartorius, that diffusion could not proceed fast enough to activate the whole of a fibre in the available time of a single twitch This conclusion is strengthened when we recall our initial assumption that the diffusing substance is not used up in reactions with the contractile material which it meets on the way; if it were, its arrival in the middle might be considerably delayed or prevented.

One of the best-known properties of muscle is the fact that the strength of a single twitch is less, often considerably less, than that of a tetanic contraction. In a frog's sartorius the ratio of twitch to tetanus may be about 1.2 at $0°$ C, about 1.4 at a higher temperature. Denny-Brown (1929, p. 377) found for cats' muscles a ratio of $1:2$ for gastrocnemius, 1.4 for soleus If now only the outside half (by volume) of a fibre was assumed to be activated in a single twitch, curve (iii) of figure 2 would be applicable instead of curve (i) and a very different conclusion would be reached. For example,

(1) for the 150μ fibre of the frog's sartorius at $0°$ C referred to above, kt/a^2 at 0.20 sec. is 0.018, for which curve (iii) gives $y/y_\infty = 0.8$;

(2) for the 100μ fibre of a frog's sartorius at $30°$ C, $kt/a^2 = 0.01$, corresponding on curve (iii) to $y/y_\infty = 0.4$; for the 50μ fibre, similarly, $y/y_\infty = 1.09$,

(3) for the 25μ fibre of the kitten's eye muscle, $kt/a^2 = 0.045$ corresponding on curve (iii) to $y/y_\infty = 1.11$. Denny-Brown's myograms (*i*) and (*j*) (figure 4) suggest that the tetanus tension of the superior rectus was in fact about twice the twitch tension.

If, therefore, in a single twitch, only the outer region of the fibre is involved, the part activated being to the whole as the twitch tension to the tetanus tension*, there would be ample time, during the observed contractile phase, for the hypothetical substance X arriving by diffusion from the surface to 'trigger' the process of contraction. The *form* of the contraction cannot be determined simply by diffusion, the contractile machinery passing over instantly to a new state as soon as X is present in high enough concentration, for the speed of development of contraction has a much higher temperature coefficient than the rate of diffusion. If the arrival of X in adequate amount at any point does determine the onset of contraction there, we are bound to assume that the local contractile process then runs its own course and completes its own cycle in its own time.

No specific theory is proposed here to bridge the gap between the processes of excitation and contraction All recent work tends to the view that excitation occurs only at the surface in muscle and nerve, and there is no reason at all for supposing that any region of a muscle fibre is not contractile. If the surface only is excited, contraction must be propagated inwards either by a process, or by a substance, travelling in. It might have been the case that no reasonable rate of diffusion could account for the known speed of contraction; and, indeed, we have seen that this would be so if the whole of the muscle fibre had to be activated in a single twitch. Since, however, we need not necessarily assume more than a part of the whole fibre to be involved in a twitch, the time required for diffusion could be much less, and propagation of contraction inwards by the diffusion of a substance would remain quantitatively possible. Indeed, the fact that the diameters of the fibres and their speeds of contraction are such that kt/a^2 comes within the range of the curves of figure 2, neither far to the left nor far to the right, might be regarded as giving a certain *a priori* probability to the view that propagation inwards is determined by diffusion. So might the fact that the ratio, twitch tension/tetanus tension, is diminished by a rise of temperature, the rate of diffusion being increased less than the speed of the contractile process.

The results of this calculation are suggestive and permissive only They merely allow us, by experiment and calculation, to examine further the hypothesis that propagation of contraction inwards is due to the diffusion of a substance liberated at the surface of a fibre during excitation. That hypothesis may be found untenable for other reasons. It is not ruled out, however, by the length of time necessary for diffusion; and it raises in a specific form the extremely important question of how the contractile process is related to that of excitation

Heilbrunn & Wiercinski (1947, p. 17) have expressed their belief that 'diverse types of stimulation cause a release of calcium ions from the surface or outer region of the cell and that this calcium then enters the cell and produces the response'.

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* [*Added in proof.*] It should, however, be noted that in a twitch there is not time enough for the full tension to be manifested externally. The contractile elements of the muscle have to shorten against the increasing tension of the elastic elements, and figure 15 of an earlier paper (Hill 1938) shows that at 0° C only about 80 per cent of the full tension is reached in 0·4 sec., when relaxation begins to set in. This necessarily reduces the apparent strength of a twitch below that of a tetanus and allows only a smaller share of the reduction to be attributed to incomplete diffusion.

APPENDIX

Throughout the space between two coaxial cylinders of radii a and b $(a > b)$, a substance X is present at time $t = 0$ at uniform concentration $y = y_0$, while inside the inner cylinder $y = 0$. The surface of the outer cylinder is impervious to X, that of the inner cylinder freely permeable. At time $t = 0$ diffusion begins · it is required to find y as a function of r and t at subsequent times.

The diffusion equation is

$$\frac{dy}{dt} = \frac{k}{r}\frac{d}{dr}\left(r\frac{dy}{dr}\right).$$

The boundary conditions are

$$y = y_0 \quad \text{at} \quad b \leqslant r \leqslant a,$$
$$y = 0 \quad \text{at} \quad 0 \leqslant r \leqslant b \quad \text{at} \quad t = 0$$

with $dy/dr = 0$ at $r = a$, and y finite at $r = 0$ at all times.

The solution is

$$y = y_\infty - 2y_0\frac{b}{a}\sum_{\alpha_1,\alpha_4}\frac{J_1(\alpha b/a)J_0(\alpha r/a)}{\alpha[J_0(\alpha)]^2}e^{-\alpha^2 kt/a^2}, \tag{3}$$

where $\alpha_1, \alpha_2, \alpha_3, \ldots$ are the positive roots of $J_1(\alpha) = 0$ and y_∞ is the uniform concentration at infinite time

Now let the zone between the cylinders become very thin, so that b approximates to a Then $2\pi a(a-b)y_0 = \pi a^2 y_\infty$, and $J_1(\alpha b/a)$ becomes $-\alpha(1-b/a)J_0(\alpha)$. Substitution in equation (3) above then gives equation (2) in the text.

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The changes induced in *Bact. lactis aerogenes* by irradiation with ultra-violet light

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Suspensions of *Bact. lactis aerogenes* have been irradiated with ultra-violet light until only a small fraction of cells survived. Strains derived from these were isolated. Their ability to grow with ammonium salts as sole sources of nitrogen was tested by measurement of the difference ΔL between the lags in liquid glucose media containing ammonium sulphate and the lags in parallel media containing asparagine and glutamic acid. The statistical distribution of ΔL values among the substrains was determined for (*a*) those derived from a standard strain by simple plating, (*b*) those derived by plating survivors from the first irradiation of the standard strain, (*c*) those derived after a second irradiation of a selected strain from (*b*). All the substrains grew easily in the asparagine-glutamic acid medium, but irradiation increased progressively the proportion which showed large values of ΔL.

The power to utilize ammonia could be restored completely to the impaired substrains by retraining.

The keto acids, α-ketoglutaric acid and oxalacetic acid, greatly decreased the lags of strains which were reluctant to grow in ammonia.

It is concluded that the ultra-violet light damages one of the key enzyme systems responsible for supplying the carbon compounds which enter into reaction with ammonia to form amino-acids.

The fact that the damage is easily reparable by growth in ammonium salt media lends support to the idea of autosynthetic enzyme systems.

The instability of the strains obtained emphasizes the desirability of quantitative measurements on bacterial 'mutants' that appear to have lost certain characters.

INTRODUCTION

The induction of mutations in organisms of definite nuclear structure by irradiation with X-rays and ultra-violet light is a well-known phenomenon. In recent years (Lederburg & Tatum 1946; Gray & Tatum 1944; Tatum 1946) it has been claimed that 'mutations' of a similar kind have been induced in bacteria by the same agencies—although the question of the existence of nuclei in bacteria is still not settled. The mutants so obtained were deficient in their ability to synthesize one or more growth factors or amino-acids. The irradiation in these experiments was usually continued until a fraction of only about 10^{-4} had survived the lethal effect, the object being to increase the chance of mutation as much as possible. The cells were then grown on complete solid media, single colonies isolated and the various strains tested in liquid media for growth requirements.

The aim of the present work was to obtain, by similar methods, strains of *Bact. lactis aerogenes* that had specific growth requirements. Since many coliform bacteria grow only with difficulty on ammonia as their sole source of nitrogen, and *Bact. lactis aerogenes* is one of the few members of the group that do so readily, loss of the ability to utilize ammonia seemed the most likely change which might result from irradiation. The experimental technique was therefore directed to find this type of

'mutant' The work of Devi, Pontecorvo & Higginbottom (1947) suggests that bacterial mutants can sometimes be very adaptable. It was thought, therefore, that a study of the stability and ease of reversion of any 'mutants' obtained would also be desirable, especially in view of the fact that a considerable amount of information about the adaptive behaviour of *Bact. lactis aerogenes* is now available.

EXPERIMENTAL METHODS

A culture of *Bact. lactis aerogenes* was grown aerobically at $40\cdot0°$ C in a synthetic liquid medium made by mixing solutions of glucose (50 g /l.), phosphate buffer (9 g./l of KH_2PO_4 and sufficient NaOH to make the pH $7\cdot12$), ammonium sulphate (5 g /l.) and magnesium sulphate (1 g /l.) in the ratios 10 . 10 : 5 . 1. When fully grown, the culture was centrifuged and washed three times in saline, the cells being finally suspended in sufficient saline to give a population of 10^9/ml About 18 ml. of this suspension were then transferred to a disk-shaped quartz vessel of thickness 1 cm., and irradiated at room temperature by a mercury vapour lamp emitting most of its energy in the range 2540 to 2800 Å (the range in which bactericidal action is greatest).

The irradiation was continued until a fraction of only 10^{-6} to 10^{-8} of the cells remained alive. This was tested in two ways·

(a) An approximate test was made by measurement of the difference Δ in apparent lag of the two cultures obtained by inoculating the same volume of irradiated and unirradiated suspension into the synthetic ammonium salt medium. Since the time T taken for the population of *Bact. lactis aerogenes* to double in such a medium is known (33 min), the fraction surviving F is approximately given by

$$F = (\tfrac{1}{2})^{\Delta/T}.$$

When the surviving fraction is 10^{-6} to 10^{-8}, Δ is of the order of 3 to 4 days. Preliminary experiments showed that between 40 and 60 min. irradiation would produce this effect.

(b) The second method was the plating out of a definite volume of irradiated suspension and the counting of the number of colonies derived.

$0\cdot1$ ml. portions of the suspension, irradiated for 1 hr., were plated out on to heart-broth agar, chosen because it is a rich nutrient medium likely to contain all the growth factors required by any possible mutants. The plates were incubated at 37° C, and when the colonies had reached a sufficient size they were transferred to 'Lemco' broth, in which they were then stored.

The lag of these irradiated strains in the simple ammonium salt medium was taken as a suitable criterion of their ability to grow in it. In order to test this adequately, however, it was necessary to compare it with the lag in another liquid medium to avoid false conclusions that mutations have occurred, when lags are really due to other causes (e.g. unsuitable age of the parent). The control medium chosen was the normal synthetic medium with asparagine and glutamic acid instead of ammonium sulphate. These two amino-acids occupy key positions in the economy of the cell and have a great ability to support growth. The experiments subsequently justified

this choice since *no strain*, whether irradiated *or* not, *ever* failed *to* grow on *this* medium in a comparatively short time.

The procedure for testing each strain was as follows: 3 loopfuls of the appropriate broth culture were transferred to a medium containing asparagine and glutamic acid, A/G, at a low concentration (0·27 and 0·38 g./l. respectively). This intervening subculture in a dilute amino-acid medium is necessary to ensure that no broth or amino-acid is carried over into the actual test medium. Under these conditions growth ceases, at a population of about 10^8 cells/ml., owing to the exhaustion of the amino-acids. As soon as growth ceased inoculations were made in parallel into an ammonium salt medium (26 ml. of medium contained 5 ml. of a 5 g./l. solution of ammonium sulphate) and into an asparagine-glutamic acid medium (26 ml. of medium contained 5 ml. of a solution with 2·5 g./l of asparagine and 2·5 g./l. of glutamic acid). The procedure may be summarized thus:

irradiated strain
in Lemco broth $\longrightarrow$ dilute A/G medium
(low stationary
population) $\bigg\langle$ $\begin{array}{l} \text{Am}_2\text{SO}_4 \text{ medium} \\ \\ \text{A/G medium.} \end{array}$
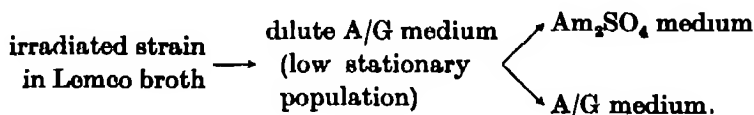
The lags were determined by the usual method. The lag in the ammonium salt medium minus the lag in the A/G medium will be designated ΔL This quantity may be regarded as a criterion of mutation in respect of the ability to utilize ammonium salts.

The above describes the procedure used to produce and test for 'mutants'; other more particular experimental methods will be described in the appropriate sections. At various stages biochemical tests (e.g the Voges-Proskauer reaction) were made to confirm that no infection had occurred.

THE EFFECT OF IRRADIATION

In order to provide a standard for comparison, the procedure described above was carried out with a culture which had not been irradiated. The ΔL values for thirty-six substrains, each derived from a single cell, were determined and the results are plotted in figure 1 *a*. The ordinate represents the percentage of the total number of strains that have values of ΔL in given ranges of 400 min represented on the abscissa. As was to be expected, the majority of the strains have values of $\Delta L < 400$ min. This group of strains will be designated S.

Experiments were then performed with irradiation of the cells in the manner already described. Figure 1 *b* shows the ΔL distribution for the thirty-one strains, S', isolated A distinct shift towards the higher values of ΔL is observed when S' is compared with S, i.e the irradiation has markedly increased the proportion of cells that find difficulty in growing on ammonium salts, in comparison with asparagine and glutamic acid.

A strain with one of the highest ΔL values was chosen from amongst the strains S' and was made the parent culture for a second irradiation experiment. The original culture was grown in an asparagine-glutamic acid medium, since the lag

in ammonia would have been prohibitively long, but this should not affect the subsequent results.

The ΔL distribution for seventeen strains S'' so obtained is shown in figure 1*c*. Compared with both S and S', displacement to even higher ΔL values is observed, though many of the strains again yield values of 0 to 800 min. corresponding to the average, unirradiated strains. The effect of a second irradiation is thus to reinforce that of the first in the sense that there are produced strains of *Bact.* lactis aerogenes that have even greater difficulty in growing in ammonia
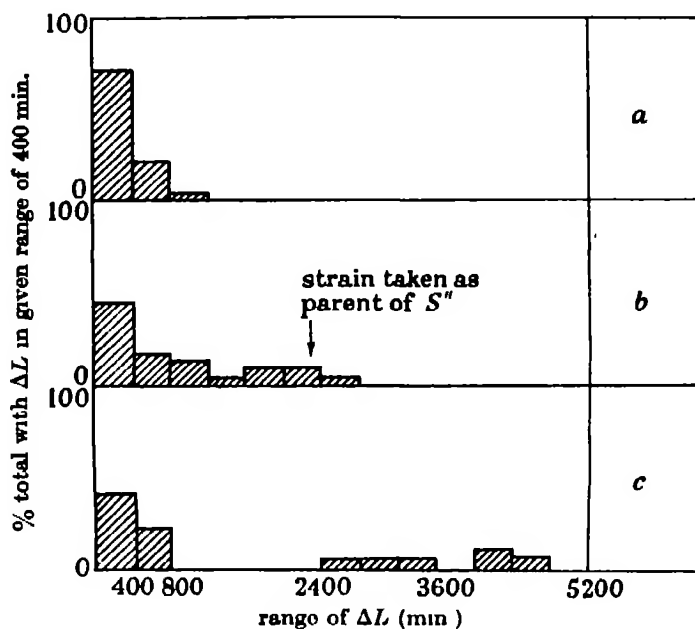
FIGURE 1

It may be mentioned here that figure 1*b* does not include five of the S' strains which at first would not grow in the dilute amino-acid medium. However, when 33 days later, the same cultures in Lemco broth were retested they grew readily in the amino-acid medium and were shown to have ΔL values of less than 800 min This question of the effect of standing in Lemco broth will be discussed again later.

THE STABILITY OF STRAINS WITH LARGE ΔL VALUES

There exists the possibility that the strains which no longer readily utilize ammonia may be retrained to do so by growth in its presence During such a process of subculturing, ΔL determinations will reveal the rapidity of any retraining.

Ten of the S' strains with the greatest ΔL values were therefore selected for a study. They were taken from Lemco broth and the ΔL values were redetermined. The cultures in the ammonium salt medium were immediately reinoculated into more of the same medium and into an A/G medium. ΔL was again determined

and the process of subculturing *in the ammonium salt medium* and of testing ΔL values was continued until ΔL became zero. The process may be represented thus.

$$
\begin{array}{c}
\text{irradiated } S' \\
\text{strain in} \\
\text{Lemco broth}
\end{array}
\longrightarrow
\begin{array}{c}
\text{dilute A/G} \\
\text{medium}
\end{array}
\underset{\searrow \text{A/G}}{\overset{\Delta L_I}{\rightleftarrows}} Am_2SO_4
\underset{\searrow \text{A/G}}{\overset{\Delta L_{II}}{\rightleftarrows}} Am_2SO_4
\underset{\searrow \text{A/G}}{\overset{\Delta L_{III}}{\rightleftarrows}} Am_2SO_4 \ldots.
$$
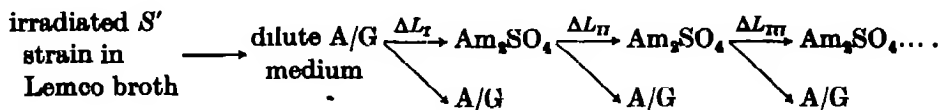
Table 1 gives the results of this experiment.

TABLE 1. THE EFFECT OF GROWTH IN AMMONIUM SALT MEDIUM ON THE ΔL VALUES OF S' STRAINS THAT ORIGINALLY DID NOT UTILIZE Am_2SO_4 READILY (ΔL VALUES IN MINUTES)

S' strain	ΔL_I	ΔL_{II}	ΔL_{III}	ΔL_{IV}	ΔL_V	ΔL_{VI}	number of subcultures in Am_2SO_4 before ΔL becomes zero
1	670	1040	50	120	0	—	4
2	545	970	980	840	700	0	5
3	530	20	0	—	—	—	2
4	2560	120	150	150	0	—	4
5	1200	130	790	340	0	—	4
6	910	460	0	—	—	—	2
7	400	215	75	60	0	—	4
8	840	680	0	—	—	—	2
9	280	320	200	330	0	—	4
10	760	903	840	320	0	—	4

Although certain fluctuations are observed—a characteristic of all lag determinations—it is clear that in an average of 3 to 4 subcultures in the presence of the ammonium salt the ability to grow with that source of nitrogen has been completely recovered. Now the inability to do so had been caused originally by the action of the ultra-violet light. These strains are therefore examples of 'adaptable mutants' —i.e. they have characteristics, induced by irradiation, that can be removed simply by growth in the appropriate medium.

A similar test with the S'' strains with the greatest ΔL values also showed that ΔL became zero after four subcultures in the presence of ammonium salt.

THE EFFECT OF LEMCO BROTH ON ΔL

One example of an effect due to the storage of cells in Lemco broth has already been mentioned. The experiments described in the last section provide another, since ΔL_I can be compared with the values originally observed (table 2).

The table shows clearly that, in most cases, ΔL has decreased considerably after the cells had been stored in broth. This phenomenon will be briefly discussed later.

<div align="center">TABLE 2</div>

S' strain	1	2	3	4	5
ΔL—1st test	> 5500	> 4600	2200	2200	1560
ΔL—2nd test after resting in Lemco broth	670	545	530	2560	1200
number of days in the broth between the two tests	22	14	22	22	14

S' strain	6	7	8	9	10
ΔL—1st test	1620	1800	1080	2125	1640
ΔL—2nd test after resting in Lemco broth	910	400	840	280	760
number of days in the broth between the two tests	14	14	14	14	14

<div align="center">TWO SUBSTANCES THAT DECREASE THE LAG OF IRRADIATED
STRAINS IN AMMONIA</div>

Morrison & Hinshelwood (1948) have examined certain strains of *Bact. coli* which grow in ammonium salts only with difficulty, i.e. they grow with long lags. There are various reasons for supposing that α-ketoglutaric acid (α-KGA) and oxalacetic acid (OAA) are of particular importance in cell metabolism. The addition of these two substances to an ammonium salt medium markedly decreased the lag of the particular *Bact. coli* strains, the first substance being the more active.

There is a great similarity between trained *Bact. coli* and normal *Bact lactis aerogenes* as regards behaviour towards ammonium salts, and also between the more resistant strains of *Bact. coli* and the strains (S' and S'') of *Bact. lactis aerogenes* produced by irradiation. It was therefore thought that the effect of addition of the same two keto-acids to some of the irradiated strains should be examined.

The strains chosen were four S'' strains with high ΔL values. After the usual subculture in dilute amino-acid medium each strain was inoculated into tubes containing the following:

(a) The usual asparagine-glutamic acid medium.

(b) The usual ammonia medium with 500 mg./l. of α-KGA.

(c) The usual ammonia medium with about 500 mg./l. of oxidized malic acid (about 30 % OAA).

(d) The usual ammonia medium.

The lags observed are given in table 3.

It is seen that the malic-oxalacetic acid mixture reduces the lag in ammonia by 50 to 60 % and α-ketoglutaric acid by 80 % or more. This behaviour is the same as that observed with the strains of *Bact. coli* already described.

<div align="center">TABLE 3. THE EFFECT OF α-KETOGLUTARIC ACID AND OF OXALACETIC ACID
ON THE LAG OF S'' STRAINS IN AMMONIA (L IN MINUTES)</div>

S'' strain	1	2	3
lag in (a), A/G	170	0	110
lag in (b), $Am_2SO_4 + \alpha$-KGA	270	40	190
lag in (c), $Am_2SO_4 + OAA$	520	190	525
lag in (d), Am_2SO_4	1240	480	1020

<div style="text-align:center">DISCUSSION</div>

As has been seen, a second irradiation reinforces the effect of the first and increases further the proportion of cells which are no longer able to utilize ammonia readily. This is strong evidence that the presence of such cells in the irradiated suspension is directly due to the action of the ultra-violet light.

The irradiation appears to have wrought an easily reparable destruction, since four subcultures in the presence of the ammonium salt are sufficient to restore the ability of the cells to utilize it as a nitrogen source. The restoration of this ability cannot be attributed to natural selection, since the damaged strain originally arose from one irradiated cell. If, however, the ultra-violet light is considered to have impaired an auto-synthetic enzyme system—i.e. a system that increases as it functions—then there is a very satisfactory explanation of this phenomenon. The damaged mechanism, the one that utilizes ammonia, is rapidly repaired by performing its function in the cell, namely, utilizing ammonia for growth.

Most coliform bacteria do not grow easily on ammonia. *Bact. lactis aerogenes* is an exception to this rule and, in one sense, its ability to grow on ammonia may be regarded as acquired—perhaps because of the exigencies of its life in the soil. From this point of view, the ultra-violet light can be considered to have destroyed an acquired ability. In fact, the whole effect of irradiation in these experiments is very similar to what has hitherto, in this laboratory, been called a reversal or loss of training.

Lemco broth contains a variety of growth factors and amino-acids and probably also ammonium compounds. The effect on the strains of storage in the broth is probably *no different* from the effect observed when they are subcultured in ammonia. It does, however, exhibit the difficulty of the maintenance of bacterial 'mutants' so as to preserve their properties unchanged

It is of interest that the ultra-violet light gave rise only to two main groups of cells; namely either dead cells, or, on the other hand, cells that grew easily on asparagine-glutamic acid but on ammonia with widely varying degrees of ability (as exhibited by the ΔL values). The damage caused by irradiation thus appears to have been either lethal or the impairment of the system that utilizes ammonia. It seems that if this latter system is intact *at all*, then, for growth in glucose, the cells seem to have no other special requirements. In other words, it has a special key position in the metabolism of *Bact. lactis aerogenes*.

Some evidence as to the nature of the system is supplied by the effects of the two keto-acids on the lag in ammonia. There is evidence from the work on *Bact. coli* that the following sequence occurs and in all probability it exists also in *Bact. lactis aerogenes*:

$$\text{glucose} \xrightarrow{A} \text{keto-acids} \xrightarrow[+\text{NH}_3]{B} \text{glutamic acid} \longrightarrow \text{other amino-acids.}$$
$$\text{(especially}$$
$$\alpha\text{-KGA and OAA)}$$
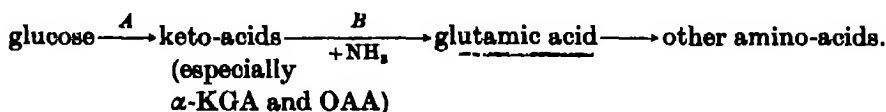
The inability of the irradiated strain to utilize ammonia could be explained by the impairment of the enzyme system catalyzing process A. The keto-acids would then

be produced only very slowly, thus causing a long lag before enough glutamic acid had been formed to allow growth at the optimum rate. The addition of the α-keto-glutaric acid and oxalacetic acid themselves to the medium would greatly reduce the lag by allowing process B to take place more readily. The lag would, however, not be reduced to zero by one keto-acid alone since several stages must be involved in B.

Complete destruction by the ultra-violet light of the enzyme system responsible for process A would lead to death, since the cells would no longer be able to utilize the best of all carbon sources, namely, glucose.

Successive cultures in ammonia (even after a long lag) would gradually bring process A more and more into operation until it could function at its normal rate. This is the phenomenon observed.

A matter of general interest arising from these observations is the following. Had the strains isolated after irradiation been tested by the technique of recording positive or negative growth in an arbitrary period such as 48 hr., what proved finally to be quantitative changes might easily have been mistaken for qualitative gains or losses of definite genic 'characters'. Definite quantitative measurements would seem to be very desirable in all such studies.

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Hearing. I. The cochlea as a frequency analyzer

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Possible methods of sensory appreciation of the nature of sounds are briefly surveyed in relation to the restriction imposed by the rate at which the nervous system can respond. It is shown that because of this restriction information must in general be lost unless peripheral frequency analysis occurs and unless the peripheral analyzer conforms to certain further conditions which are enumerated. One of these conditions is that the selectivity of the resonant elements shall be proportional to frequency.

Experimental evidence is submitted that the selectivity of the resonant elements even in the upper half of the auditory spectrum of the human ear is in fact roughly proportional to frequency and is very much higher than has generally been supposed.

Previous theories of hearing are considered, and it is shown that only the resonance hypothesis of Helmholtz interpreted in accordance with the considerations enumerated in the first part of this paper is consistent with observation.

In particular the experimental data which have been supposed to be evidence of high damping of the cochlear resonators are re-examined. It is shown that they are either irrelevant or that, correctly interpreted, they are evidence for the contrary view.

Finally, an attempt is made to summarize some important properties of the ear in a diagram which illustrates that the ear is a perfect analyzer up to a frequency of 1 kc /sec. Above that frequency it is imperfect, not because of inadequate selectivity, but because perfection would require an impracticable number of resonant elements and nerve cells.

1. The theory and evolution of peripheral analysis

It is the function of the ear to convey to the brain a maximum of relevant information contained in the sound that reaches it. That information may include the material for a judgement of direction, distance and nature of the source. We shall concern ourselves here only with processes involved in the recognition of the nature of the sound, and not deal explicitly with the judgement of direction and distance, though it should be appreciated that the latter has also been a determining factor in the evolution of the ear. As far as the recognition of the nature of a sound is concerned it should also be appreciated that the requirements of different animals are very different, and that a simple classification of peripheral mechanisms according to quality is hence not possible.

Apart from directional effects a sound is always completely described by the variation of air pressure with time. We may well ask, then, why ears do not simply function by generating nerve signals corresponding to the instantaneous pressures. If such signals were faithfully conveyed to the brain, then there would be no loss of information. The action of such an ear would be similar to that of a microphone.

The reason why such a mechanism is not generally used is to be found in the limitation of the rate of action of the nervous system The maximum rate of response of nerve fibres is related to the rate of response of other tissues and the rate of living of the animal as a whole. The maximum rate at which nerve signals can be set up is a few hundred per second, and the rate of acceptance by the brain of discrete items

[462]

of information in one channel is in all probability still much lower. It follows that if an animal is to obtain a substantial amount of intelligence from the rapid pressure-time variations of a sound, a peripheral transformation to a multi-channel system must be effected. The pressure-time variation must be coded in such a way that each nerve fibre only carries an assigned fraction of the information, and this transformation must occupy a negligible interval on the time scale of the animal if the greatest benefit is to be derived.

Different types of transformation are possible and are actually found to occur. No one of these is so perfect that all the information of an arbitrary sound signal can be accepted, and in practice the type of transformation adopted is evidently dependent on the evolutionary history, and the size, habits and structure of the animal under consideration. Even if the animal is content to neglect the fine detail of the pressure-time variation and accept only the intelligence contained in pressure fluctuations as slow or slower than the maximum response rate of its nerve fibres, a transformation to a multi-channel system is necessary to make intensity discrimination possible over a wide range. Simple ears of this type are known to occur in insects and are probably widespread in the animal kingdom.

Another possibility is for the peripheral mechanism to signal to the brain the mean acoustic power which it absorbs in successive short intervals of time. In such a system the detailed information about rapid variations of pressure is lost, but an appreciation of intensity variations can take place with the best time discrimination of which the nerves and brain are capable. This is the principle of the tympanic organs of insects, whereby they are enabled to hear and discriminate between sounds whose frequency components lie mostly between 10 and 100 kc./sec., provided that the mean intensity of such sounds, averaged over intervals not much shorter than $\frac{1}{100}$ sec., fluctuates in a characteristic way (Pumphrey 1940). In such an ear the band of frequencies from which information can be extracted is limited only by the characteristic of the pick-up mechanism, but the information is distributed to the nerve channels on a basis of intensity only. As in the previous example many channels are necessary for intensity discrimination over a wide range of intensities.

In mammalian ears the problem of accepting items of information more rapidly is solved by the device of frequency analysis. It is of course possible, in principle, to analyze any wave form into frequency components in such a way that very little information is lost. This always implies that the recognition of frequency has to be good if rapid changes cannot be recognized. In the mathematical limit such an analysis becomes a Fourier analysis when the input signal (a function of time) is completely described by the output which is a function of frequency only.

The particular analysis which is adopted in mammalian ears lies between the two extremes of an instantaneous recognition of the signal fluctuations and an indefinitely accurate recognition of the frequency components. Our aural sensations are determined by the precise nature of this compromise, and hence represent only one of many possible frequency analyses.

A physical system which is capable of such an analysis is one possessing a large number of resonant elements of staggered natural frequency. A knowledge of frequency can then be derived from the identity of the resonator which responds

most, or from some comparison of the responses of different resonators. Helmholtz (1877) supposed the mammalian inner ear to be a system of this kind and, as we shall see, *this view is amply substantiated by experimental evidence.*

At this stage it will be of interest to consider the properties which such a physical device must have, and for simplicity we shall consider a system of mutually independent resonators, each supplied with a device by which its displacement amplitude may be measured. The displacement amplitude of one element in response to a signal of the form $F \sin \omega t$ *(a continuous pure tone) is then given by*

$$A = \frac{QX}{\frac{\omega}{\omega_0}\sqrt{\left\{1 + Q^2\left(\frac{\omega}{\omega_0} - \frac{\omega_0}{\omega}\right)^2\right\}}},$$
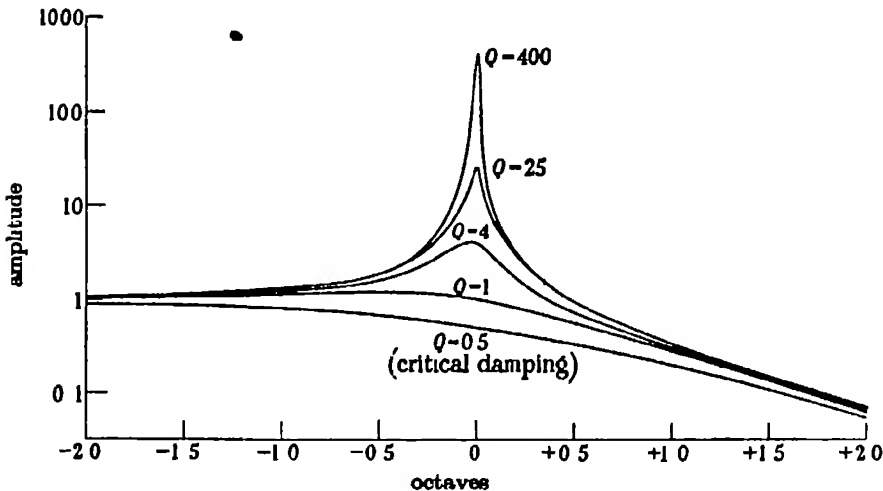
FIGURE 1

where ω_0 is the resonant frequency of the element, ω is the applied frequency, X is the displacement amplitude which a steady (non-oscillatory) force of magnitude F would produce, and Q is the usual parameter defining the degree of resonance. Figure 1 is a graphical representation of this formula, and shows the amplitude plotted against frequency. The frequency is shown in octaves away from the natural frequency of the element, and the amplitude is also plotted to a logarithmic scale. The curves for different values of Q correspond to resonant elements which are similar in all respects except in their degree of resonance. By convention systems of $Q > 25$ are regarded as highly resonant (or lightly damped).

We see that the amplitude at resonance is Q times as great as that which a non-oscillatory force of similar magnitude would produce.

The time response of an element of natural frequency ω_0 is best described by its response to a sudden beginning or end of a wave of the form $F \sin \omega_0 t$. The envelope of the oscillations is then given by

$$A = QX(1 - e^{-\omega_0 t/2Q})$$

for the rise from the quiescent state, and by

$$A = A_0\, e^{-\omega t/2Q}$$

for the decay from an amplitude A_0 (figures 2 and 3).
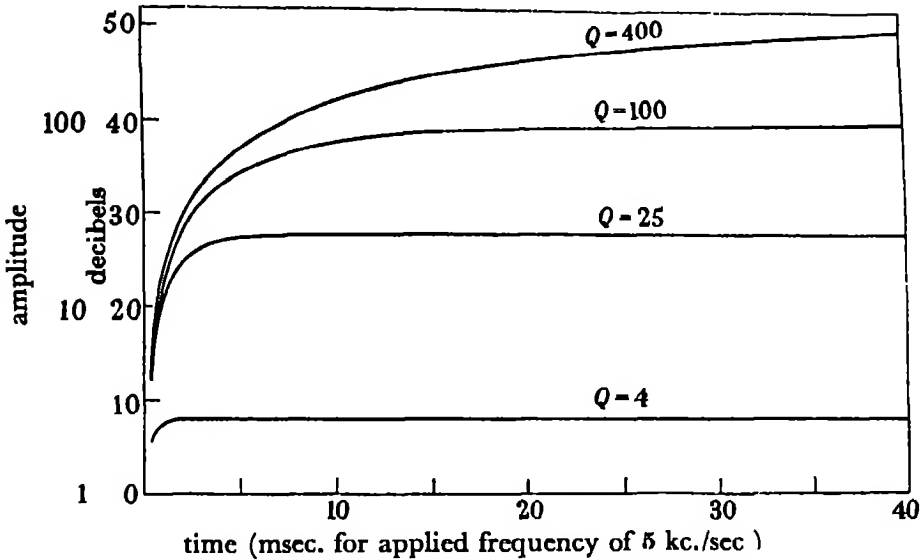
FIGURE 2
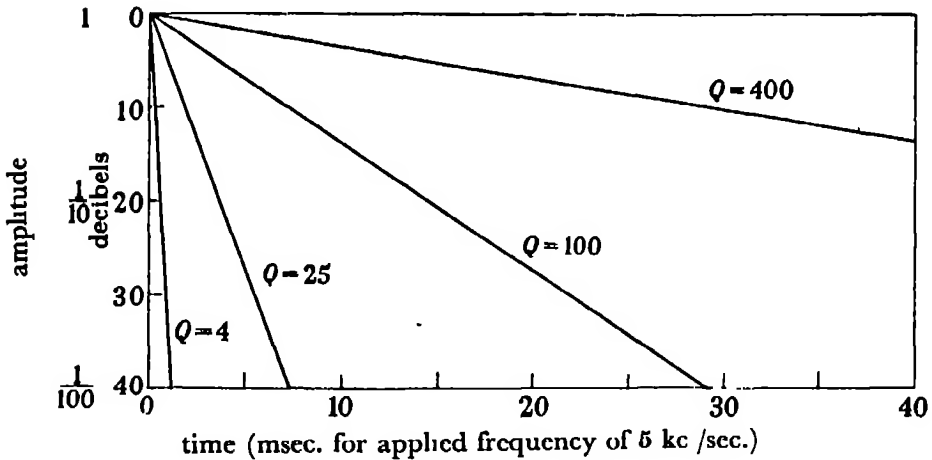
FIGURE 3

The following conclusions may now be drawn; they are accurate for all except very low values of Q.

(1) A resonant element specified in all respects except its degree of resonance will suffer a maximum displacement for a given force, when that force is applied at the natural frequency of the element, in that case the displacement will be proportional to the value of Q. Similarly, the power required to produce a given displacement is proportional to $1/Q$.

(2) The range of frequencies over which the response of an element is less than the maximum by a given fraction is narrower the higher the value of Q.

(3) The initial rise of oscillations in response to a sudden beginning of a wave is independent of the value of Q. The time taken to approach the steady-state amplitude to a given approximation is proportional to Q/ω_0.

(4) The time taken by free oscillations to decay by a given factor is proportional to Q/ω_0.

(5) The response to a force applied at a frequency far removed from the natural frequency of the element is nearly independent of the value of Q. If the force is applied at a much higher frequency, the response decreases by a factor of 4 every time the frequency is doubled; if the force is applied at a much lower frequency the response tends to a constant value, namely, that which a steady force would produce.

If we are now to consider the performance of any physical frequency analyzer responding to the displacement of its elements, then we have to specify the relevant technical limitations. First, the overall acceptance band of the analyzer is finite. Secondly, the number of resonant elements covering this frequency band is finite, and the band can hence not be covered perfectly smoothly The number of elements required to cover the band smoothly to a given approximation will be greater the higher the value of Q of the elements Thirdly, the recognition of displacement cannot be indefinitely accurate. Displacements of less than a certain amount cannot be recognized at all, while displacements greater than that amount can only be measured with a finite accuracy. Lastly, we must specify a limitation relating to the recognition of time interval If a change of amplitude of a resonator occurs in an interval which is shorter than a certain amount, then the length of this interval cannot be recognized, i.e there is a finite time constant associated with the recognition of amplitude.

In order to assess the dependence of the performance of such an analyzer on the values of Q let us begin by considering one composed of low Q elements. It is clear that an increase in the value of Q will be entirely beneficial, provided that the number of elements is great enough to cover the band sufficiently smoothly, and provided that the resulting increase of the decay time of each element is still swamped by the time constant introduced in the recognition of its amplitude. The effect of such an increase of Q will be first to cause a proportionate increase in the threshold sensitivity and secondly to increase the accuracy of recognition of frequency. Only when the values of Q are high enough for the two time constants to become similar will a further increase have a detrimental consequence; the time discrimination will then become poorer, and will eventually be limited by the decay time of the resonant elements.

The frequency analyzer of mammalian ears will be subject to similar considerations, although, as we shall show later, the assumption of complete independence of the elements is not quite justified. It will in this case, again, be purely of advantage to the species if the value of Q of each resonant element is increased, provided that an increase in the number of elements can be afforded so that the relevant frequency band remains smoothly covered. The effect will be an improvement of frequency discrimination, and at the same time an increase of sensitivity to prolonged tones.

It is difficult to compare the relative importance of these two qualities to the evolution of the ear, but the fact that they demanded similar and never conflicting trends of development must be held largely responsible for the perfection achieved with respect to each.

If we wish to investigate what values of Q would result finally from an evolutionary process, assuming that any can be achieved technically, then there are two important considerations. First, there is the requirement of time discrimination and the appreciation of the instant, any infringement of this will be disadvantageous. And secondly, there is the consideration of the types of natural noises to which the ear was required to be sensitive. We will deal separately with these two factors which limit the useful value of Q, although we suppose that the compromise reached in the evolutionary process will have been influenced by both at the same time.

The conflict with time discrimination will arise when the stage is reached that the decay time of an element becomes comparable with time intervals which can be appreciated by the nervous system. A further increase of Q would make the decay at the end of a signal subjectively recognizable, rapid successions of transients would be drawn into each other and their identification hindered, loud noises would mask subsequent weak noises. The response to a sudden beginning of a signal would of course not suffer, though the time taken to reach a steady state might also become recognizable. The question will hence arise whether it is better for the species to improve further their facility for the detection and analysis of prolonged tones, and at the same time to deteriorate with respect to time discrimination, or whether the recognition of the instant and of short intervals should take place with the best definition of which the nervous system is capable.

The question of the character of the noises which determined the evolutionary history of the ear is very relevant in this connexion The diversity of such noises prevents us from using any precise quantitative definitions, and a qualitative treatment of this point will have to suffice. Let us for this purpose divide noises into two classes: first, those which are principally a combination of some 'pure' tones, so that most of their energy is concentrated in a few narrow regions of the spectrum; and secondly, those which are principally a combination of transients, so that they have no sharply defined frequency components, but have their energy widely distributed over the spectrum. For the reception of the first type a frequency analyzer composed of high Q elements is of advantage for the reasons outlined above. But for the reception of the second type of noise, such high values of Q would be unnecessary, though not detrimental If all the spectral maxima of a sound are wide, then no definition will be lost, provided the frequency response of the analyzer elements is not quite so wide, elements of much sharper tuning would result in no improvement of sensitivity or discrimination These considerations lead to a restriction of the value of Q; it would be limited to that value where the marginal gain of an increase becomes insufficient to warrant the corresponding increase in the number of elements required.

Most incidental natural noises fall into the second class, the impact of stones or the cracking of a twig produces a sound whose energy is rather diffusely spread over the spectrum, though there may be some peaks corresponding to the natural modes

of oscillation of these objects. Such noises would not set a very high limit to the useful value of Q, that value would, in fact, be of the same order as the values of Q which could be assigned to the natural oscillations of the objects concerned.

The deliberate sounds made by mammals, however, fall largely into the first class of noises. There a fairly strict periodicity may persist for seconds, so that an appreciable fraction of the energy may be contained in a number of narrow spectral regions. With respect to such noises a much higher value of Q would be useful, and we may hence suppose that the perfection of the auditory frequency analyzer of a species will have gone hand in hand with the development of its own vocal organs in the first place and in the second with the vocal organs of other species in which it may come to be interested. In particular, it may be of importance to a species for individuals to keep the intensity of their calls to one another as low as possible. It will then serve them well to have a region in their hearing range where the Q is particularly high, and to use corresponding calls of a rather pure spectrum. Considerations of this sort may fix a very high upper limit to the useful value of Q; whether under those conditions a value ever results which infringes on time discrimination is not known, it is perhaps unlikely, for, other things being equal, it would always involve less sacrifice to choose a frequency for communication so high that Q can be high without making the time constant $Q/\pi f$ long enough to affect time discrimination adversely. It is significant that the male voice of mammals commonly becomes low and rough and loud, while communication between mother and offspring remains at a high frequency and high purity level. We would, however, suggest that in general amongst mammals the two factors limiting Q, namely, the insufficient marginal gain and the requirement of time discrimination, would not lead to greatly different values. This is saying no more than that an animal is likely to be most interested in noises whose frequency components fluctuate about as fast as it can appreciate such fluctuations, a connexion which is evident in the case of speech, where the speed of fluctuation of frequency components and their reception are subject to similar limitations.

If mammalian ears had progressed entirely to the point where they just avoided infringement of time discrimination, then the time constant of all resonant elements should be a certain fraction of that of the nervous system; the value of Q of each element should hence be proportional to its natural frequency, and the number of resonant elements per octave should also be proportional to frequency. These conditions are approximately fulfilled in the middle of the human auditory spectrum. A departure at low frequencies can be ascribed to the necessity of employing longer time constants if frequency analysis is to be achieved (see concluding section of this paper). And where the condition is not fulfilled at high frequencies, we suggest that insufficient marginal gain and economy of elements have been the principal reasons.

When we considered the threshold sensitivity, we defined the limitation as being entirely one of recognizing displacements larger than a certain value. As it has frequently been suggested that the sensitivity of the human ear comes close to the limit dictated by thermodynamic considerations, it is worth pointing out that this would in no way affect the arguments presented.

If we are dealing with independent resonant elements of all but the lowest values of Q, then the mean thermal energy stored in the degree of freedom with which we are concerned is given by $\frac{1}{2}kT$ (where k is Boltzman's constant and T the absolute temperature). The displacement corresponding to that value of the energy is given by the mass and the natural frequency of the element, and is independent of the degree of resonance. The threshold level would now merely correspond to a displacement which is sufficiently greater than that due to the thermal effect, so that the statistical fluctuations would generally be inadequate to reach it.

If we define that the energy stored in the resonator at threshold shall be n times the mean thermal energy, then we obtain for the threshold power of a pure tone at resonance

$$P = \frac{\omega n k T}{Q},$$

which is a well-known formula. The power required to activate the device which measures the displacement (the detector) does not appear in this formula, this power can, in fact, be arbitrarily small, depending only on the technical perfection.

We see that under those conditions the threshold power is again inversely proportional to Q.

2. THE EXPERIMENTAL DETERMINATION OF THE SELECTIVITY OF THE RESONANT ELEMENTS OF THE HUMAN EAR

This section of the paper consists of two related subsections. The first is concerned with the determination of a time constant of the ear at various frequencies. In the second, a proof is given that this time constant is actually that of the resonant elements and is consequently a measure of their selectivity.

It will be noted that the truth of the proposition proved in the second subsection is assumed in developing the argument of the first, that is to say, we write of the determination of selectivity throughout the first subsection. This is done solely to avoid unnecessary circumlocution and evidently introduces no logical error.

Subsection I

The experiment described in this subsection allows us to arrive at a more accurate determination of the selectivity of the ear at chosen frequencies than has hitherto been possible, by a comparison of the subjectively determined thresholds for 'transients' and for 'continuous pure tones'. Both these expressions need definition in psychological terms, for neither can be used in the accepted abstract sense. In nature all sounds are transient, since they have a beginning and an end. For the purpose of our experiment we define a 'continuous pure tone' as a sinusoidal pressure variation of the air of greater than threshold intensity, and enduring so long that a further increase in duration does not perceptibly alter its attributes of pitch and loudness, and we define the type of transient, which we shall consider, as a pressure variation of similar character, but of less duration than a continuous pure tone.

It is already known that there is a transition in perception from a transient to a continuous pure tone, but it is gradual, and the point of transition cannot be

determined accurately for reasons which will become obvious as the present argument is developed. At any particular frequency it is, however, possible to find a duration for less than which a tone has certainly a transient character, and another greater duration above which a tone has certainly the character of a continuous pure tone, and this fact alone allows upper and lower limits to be set to the selectivity of the ear at any chosen frequency, and contradicts assertions that the responsive elements of the inner ear are critically damped.

We shall show that the selectivity can be determined with much greater precision by comparing the threshold for a continuous pure tone with the threshold for short transients.

Consider first the action of a transient oscillatory force on a simple resonator of resonant frequency f_0. Let the force have the form $F \sin 2\pi f_0 t$ for n cycles and be zero before and after. Then the maximum amplitude of oscillation of the resonator is

$$A = A_\infty \left[1 - \exp\left(-(n - \tfrac{1}{4}) \frac{\pi}{Q} \right) \right], \tag{1}$$

where A_∞ is the amplitude for very large n and Q is the parameter defining the selectivity of the resonator *

We can rearrange expression (1) as follows:

$$P_n - P_\infty = -20 \log_{10} \left[1 - \exp\left(-(n - \tfrac{1}{4}) \frac{\pi}{Q} \right) \right], \tag{2}$$

where P_n and P_∞ are intensities measured in decibels with respect to an arbitrary intensity level, P_∞ being the intensity required to produce a particular amplitude of oscillation in a very large number of cycles, and P_n the intensity required to produce the same amplitude in n cycles

If we plot $P_n - P_\infty$ against n, or more conveniently $\log_{10} n$, we obtain a family of curves each corresponding to an assigned value of Q (figure 4.) If, therefore, we can excite the resonator with transients for any desired value of n at any desired intensity, and if we have a means of knowing whether the amplitude of the resonator is or is not greater than a particular amplitude, we can determine Q for the resonator with an accuracy depending only on the accuracy with which we can determine $P_n - P_\infty$. We can now apply expression (2) to human hearing, equating P_n and P_∞ with the subjectively determined thresholds for a transient of n cycles duration and for a continuous pure tone respectively, but in so doing, certain assumptions must be made and certain experimental conditions fulfilled. The experimental conditions

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* The behaviour of an electrical resonator is defined by the equations $\omega_0^2 LC = 1$ and $Q = \sqrt{(L/CR^2)}$. For small (and therefore linear) displacements of a mechanical resonator, the same equations apply substituting mass for inductance and compliance for capacity. The resonant frequency $\omega_0/2\pi$ is that frequency at which the impedance of the resonator is real and the velocity is in phase with applied force. The frequency of maximum displacement amplitude is $\dfrac{\omega_0}{2\pi} \sqrt{\left(1 - \dfrac{1}{2Q^2} \right)}$, and the frequency at which the resonator 'rings' when the applied force is removed is $\dfrac{\omega_0}{2\pi} \sqrt{\left(1 - \dfrac{1}{4Q^2} \right)}$. These frequencies are evidently nearly equal to the resonant frequency if $Q \gg 1$.

are, first, that the transient shall be accurately of the form $0 \mid F \sin 2\pi f_0 t \mid 0$, and that the displacement shall be everywhere continuous, that is, that $2n$ shall be an integer. Secondly, it must be remembered that when n is small, the frequency spectrum of transients such as we have been considering shows prominent maxima at frequencies widely displaced from the fundamental frequency f_0. In applying expression (2) to
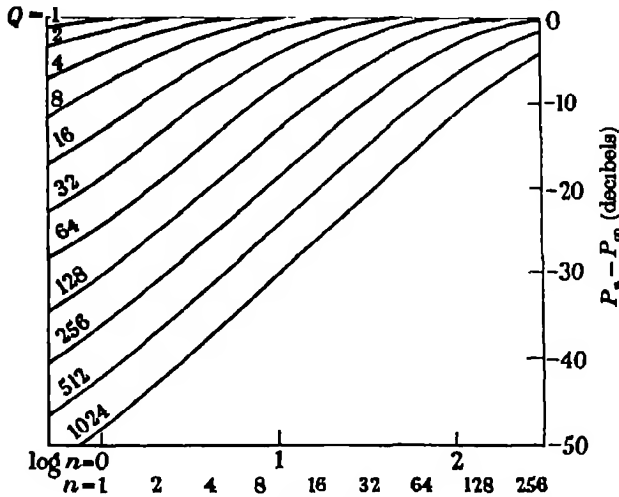
FIGURE 4
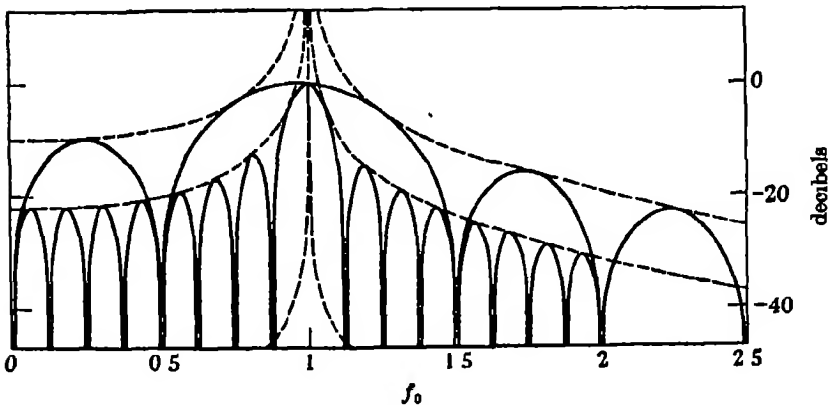
FIGURE 5. The continuous lines represent the Fourier integral for pulses of 2 and 8 cycles duration at frequency f_0. The energy is expressed on a decibel scale with reference to the energy of the f_0 component. The dotted lines represent the envelopes of pulses of 2, 8 and 512 cycles respectively.

the ear f_0 must be chosen so that the threshold at f_0 is not much higher than at frequencies corresponding to the other maxima. (To illustrate this point the Fourier integrals for certain values of n are plotted in figure 5.) In practice this means that f_0 must not lie in the region of the spectrum below 800 c./sec., where the threshold is falling rapidly with increasing frequency if unambiguous results are to be obtained with small values of n. Reference to figure 4 shows that n must be small (unless Q is

large) if $P_n - P_\infty$ is to be measurable, since 1 db. is the limit of accuracy for subjective threshold measurement.

The assumptions we make are covered by the basic assumption that the ear works in the simplest possible way consistent with its known performance. More specifically, we assume (1) that the cochlea contains an array of linear* resonant elements ranged in serial order of resonant frequency, and that these elements are the agents which individually excite those hair cells of the organ of Corti which are in apposition to them, (2) that the difference in resonant frequency between two adjacent resonant elements does not exceed the difference in frequency which can be subjectively discriminated at the same point on the frequency scale; (3) that a sound is heard when at least one resonant element exceeds a displacement amplitude of oscillation which we call the threshold amplitude.

The first of these assumptions is the *raison d'être* of our experimental approach. It is a brief statement of the resonance hypothesis, and it will be shown that our results and others are consistent with it only. No further justification is needed.

The second assumption follows quite logically from a consideration of data on pitch and intensity discrimination, for, if the selectivity of a resonant element were sufficient to permit frequency discrimination of the degree actually found, and the spacing of the resonant frequencies were greater than the limit assumed, it is evident that the plot of threshold intensity against frequency would show a series of minima and maxima corresponding respectively to the resonant frequencies of successive elements and to frequencies falling in the trough between them, and this does not happen.

The third assumption is more questionable. The threshold intensity for continuous tones is known to vary on a long-time scale for reasons which cannot always be determined. Our procedure, however, ensures that it is sensibly constant over the period of an experiment. It is much less certain whether the threshold amplitude on a short-time scale depends on the rate at which it is reached. As no data are available, we make in the first instance the simpler assumption that it does not. One aspect of this point is treated further on p. 474.

Equipment and procedure

The crucial parts of the equipment are the electronic circuits for generating transients of the desired form and the transducer for converting faithfully a voltage variation into sound. It is unnecessary to give all the circuits in detail since they conform to standard practice, but the mode of operation must be briefly described.

A sine-wave generator, whose output is variable in frequency over the audible band and constant in amplitude, 'locks' a relaxation oscillator so that each relaxation occurs in a fixed phase relation to the sine-wave output. The output of the relaxation oscillator consists of brief voltage pulses at a repetition rate of about 4 per sec., and each pulse initiates a cycle of activity in two square-wave generators' arranged so that the second square wave begins when the first ends. The second

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* The assumption of linearity is always justified for limitingly small displacements, and *at threshold* the displacements are certainly small enough since the ear shows little evidence of non-linearity within 20 db. of threshold.

square wave holds open for its duration an electronic gate. The gate permits the output of the sine-wave generator to pass when it is open but not when it is closed. The pulses of the relaxation oscillator also initiate a very linear time base applied so as to produce a horizontal trace on a cathode-ray tube, while the output of the' gate is applied so as to produce a vertical deflexion. A stationary picture of the output when the gate is open is consequently visible on the cathode-ray tube and can be continuously monitored. The duration of the two square waves referred to above are independently controllable. The cycle of operations will be clear from an examination of figure 6   Provision is made for leaving the gate open indefinitely when required. The output of the gate is led through an attenuator with a range of 108 db. in 2 db. steps to an output amplifier feeding the transducer.
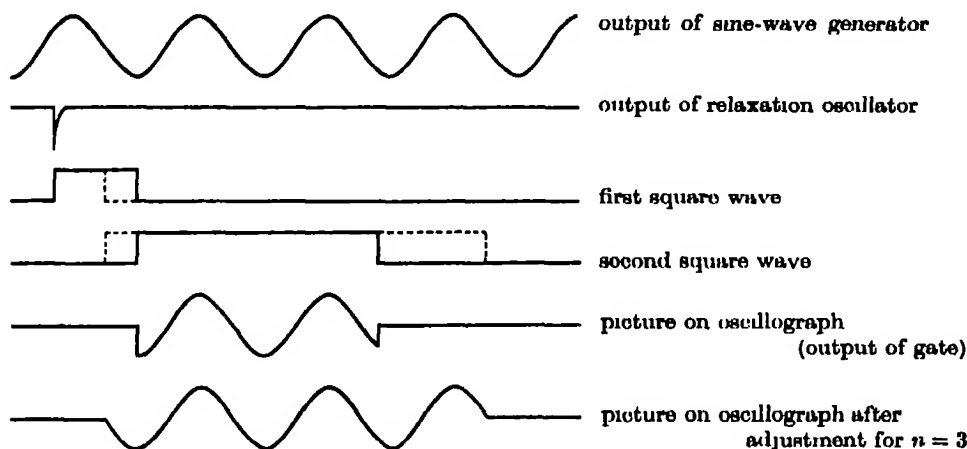
FIGURE 6

The transducer consists of a Rochelle-salt pack mounted in a block of paraffin wax. The pack (obtained from a piece of service equipment) consists of fifteen plates each ⅜ in. square by 1 mm. thick. It has a nominal resonant frequency of 100 kc /sec., and we failed to find evidence of resonance in any mode at lower frequencies. It may, therefore, be assumed that it is practically dead-beat at audible frequencies. It is, of course, extremely inefficient, because it is a very bad match to the air.

The transducer was mounted in a sound-proof cabinet with absorbent walls (approximately a 7 ft. cube), so that the subject of the experiment could sit comfortably with one ear directed towards and on the axis of the transducer and about 12 in from it. The subject's head had a support but was not clamped, though he was requested to keep it still while a series of observations was being taken. He was given a push-button connected with a lamp outside the cabinet, and requested to press it when and as long as he was sure he could hear a sound

In taking readings our practice was to increase the intensity gradually until the signal light came on, then decrease it and repeat the process until a setting was found which consistently elicited a response from the subject while one 2 db. lower in intensity did not. We then took the higher reading as the threshold. If, as rather

less frequently happened, a setting was found to which the subject sometimes, but not always, responded, we assessed the threshold as 1 db. higher than this setting.

A series of readings was always begun with a threshold measurement for a continuous tone (with the gate open), followed by a threshold determination for a transient of a chosen number of cycles, and these measurements were alternated until at least three successive determinations of each threshold agreed within 2 db. The alternation of continuous-wave and transient threshold measurements was then continued for other values of n, at least two consistent successive readings being taken for each value of n and the series being discontinued if the continuous-wave threshold showed any tendency to drift.

It takes rather a long time to get any number of consistent readings, since most subjects show a more or less regular fall of threshold at the beginnings of a run and a considerable irregularity when they are tired, and we have ignored readings taken when the continuous-wave threshold was inconstant.

Results

Because of the laborious character of the experiments, especially with inexperienced subjects, we have obtained most of our results with one subject (W.S.D.), whose hearing in both ears was good. We have, however, taken enough readings with other subjects (including ourselves) to be satisfied that our results with W.S.D. are representative. These are reproduced in figure 7, in which the constant Q curves as in figure 4 are also shown to give a frame of reference. It will be noted that the agreement in slope at any one frequency between the points and one of the Q curves is good, the deviations being most marked when $P_n - P_\infty$ is small and the error of measurement proportionately large, and also for the points when $n = 2$. In this case, the deviation is in the sense expected if the ear is first excited by the side bands of the pulse and is most marked for the lowest frequency as predicted.

It is pertinent to inquire whether there are any sources of systematic error which we have not yet considered. Our experience of making these measurements suggests one such possible source of error in that the thresholds we are measuring may not be strictly commensurable for pure tones and for transients. We find in practice that the measured threshold for continuous tone differs by about 1 db. according to whether the intensity is increased by turning the attenuator knob rapidly or slowly, the former giving the lower threshold. We attribute this difference to the greater obtrusiveness in consciousness of a rapidly changing stimulus without speculation as to its origin, though it could be attributed simply to peripheral sensory adaptation. As a result of this observation we might conclude that a sequence of transients would have greater obtrusiveness than a continuous tone, and that 1 or 2 db. should be added to the values of $P_n - P_\infty$ which we have plotted. Even if we allow a factor of 3 db. for this error it would only mean the true value of Q exceeded our uncorrected estimate by a factor of $\sqrt{2}$. Without making this allowance, table 1 shows in round figures the values of Q for different frequencies in our range. Comparison with two other subjects indicated that the high value at 10 kc./sec. is abnormal and that the average would be nearer 200. The rather steep fall in Q for frequencies below 2·5 kc./sec. seems to be quite typical, as is, indeed, to be expected both from

frequency-discrimination data and also because a high Q at low frequencies would hinder the subjective appreciation of loudness fluctuations. As Helmholtz pointed out, a trill or a run in the bass sounds blurred for reasons which are not principally instrumental. A resonator of a natural frequency of 50 c./sec. would need to be
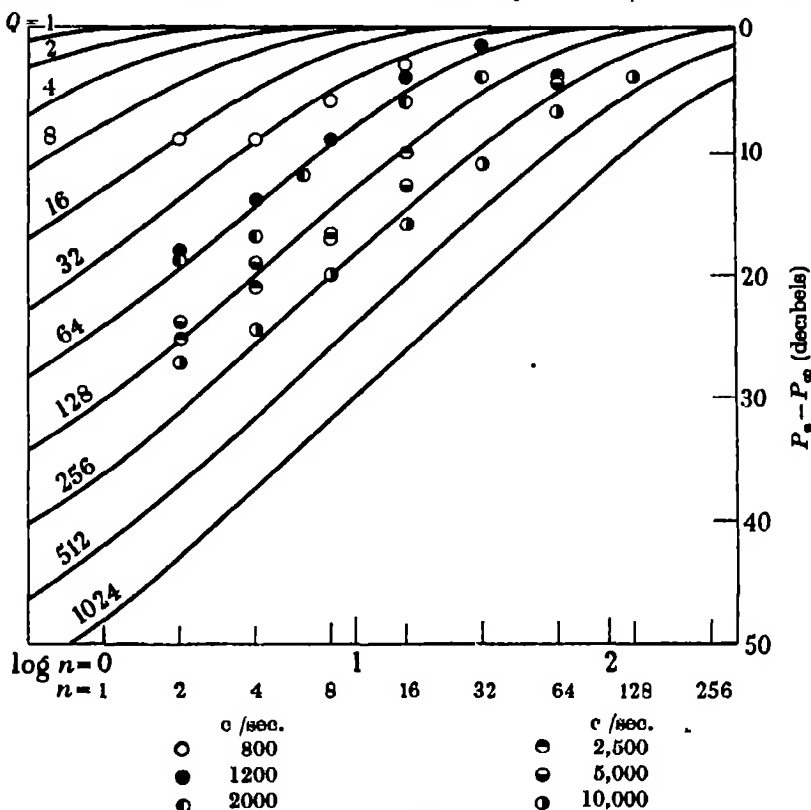
FIGURE 7

TABLE 1. W.S.D. AGED 30

frequency (c./sec.)	Q	time constant $= Q/\pi f_0$ (msec.)
10,000	300	10
5,000	150	10
2,500	150	19
2,000	80	12
1,200	60	16
800	32	13

nearly critically damped if its amplitude is to die down so quickly as not to overlap the next note. Because the damping of the human ear even at the lowest frequencies is less than critical, some blurring does occur and the intelligibility of speech is actually improved by a bass cut.

Comparison of the values of Q obtained from W.S.D. with the audiometer curve for the same ear showed that the high value of Q at 10 kc./sec. corresponded with

a marked maximum of sensitivity at 9·8 kc./sec. It is perhaps significant that this subject when reproached for inattention after a hopelessly inconsistent series of readings had been taken, complained that the test stimulus was masked by 'singing in the ears'. On further inquiry, he stated that spontaneous tinnitus was a common experience always taking the form of a clear 'ring' of constant intensity and well-defined pitch.

Subsection II

Although we have assumed in the preceding subsection that we were determining the selectivity of the resonant elements of the ear, it could be argued that we were, in fact, measuring a time constant associated with some other part of the sensory process, e.g. the time constant of excitation of the nerve endings associated with the hair cells. Put more generally our experimental results in the first subsection could be stated as follows. At a given frequency a sound pulse must have a certain energy in order to be heard if its length is less than a certain time, whereas it must have a certain intensity to be heard if it is longer than this period. And, as a non-resonant system which would have this property is conceivable, our results do not necessarily justify the attribution of specific properties to the resonators of the ear. Such an argument is inherently implausible because it implies that the ear is less efficient than it could be (see discussion in § 3). Nevertheless, it was desirable to carry out an experiment which would discriminate firmly and unambiguously between these possibilities, viz. that the ear is a frequency analyzer composed of high Q elements or that it is composed of low Q elements, the long-time constant being introduced after analysis.

The experiment we performed was technically elaborate and its detailed analysis is complex. But the principle of the experiment and the conclusion to be drawn from the result can be simply presented

A subject was required to listen alternately to two auditory stimuli and to say whether they were the same or different. One stimulus consisted of a train of oscillatory pulses of the form $F \sin 2\pi ft$ and n cycles duration, each pulse being separated from the next by a silent interval of length corresponding to m cycles at the same frequency, n and m being integers. The other stimulus was identical with the first in all respects save that the phase of every other pulse was reversed. The energy of every pulse in both stimuli was therefore the same both in magnitude and spectral distribution.

If the subject succeeds in discriminating between the two stimuli there are two and only two possible explanations. Either the longest time constant of the sensory process is so short that the direction of the displacement in the initial half-cycle of every pulse can be appreciated, or the time constant of the resonant elements is so long that their free oscillations have not fallen to an insignificant amplitude before the next pulse.

The first explanation can be excluded both on general grounds and because, if it were true, the subject's ability to distinguish between the two stimuli should be independent of the length of the silent interval, whereas in fact it falls steeply with increasing m.

We actually find that the distinction can be readily made even by subjects with no training. For example, at a frequency f of 5 kc./sec. and a pulse length of 10 cycles at an intensity level of about 20 db. above threshold, the difference between the two stimuli was glaringly obvious with a silent interval of 10 cycles and was still perceptible though with greater difficulty when the silent interval was increased to 30 cycles.

The energy in a resonator when the applied force is removed falls at the rate of π/Q 20 $\log_{10} e$ db./cyc. or approximately 27/Q db./cyc If Q for the resonant elements in the neighbourhood of 5 kc /sec. were low, say, 10, it is evident that the residual energy in the elements would have fallen below threshold in less than 10 cycles of the silent interval and after 30 cycles would have been more than 40 db. below threshold, and it would consequently have been impossible for a subject to distinguish the signals. The only tenable explanation is that $Q > 60$ at 5 kc./sec., probably substantially greater. This is consistent with the interpretation we have adopted of the results in the preceding section.

Equipment and procedure.

As in the first subsection, it is essential to generate electrical transients of the desired form and to convert them accurately into sound. In this case, however, it is easy to show that the detectable difference between the two alternative stimuli is not due to instrumental imperfection.
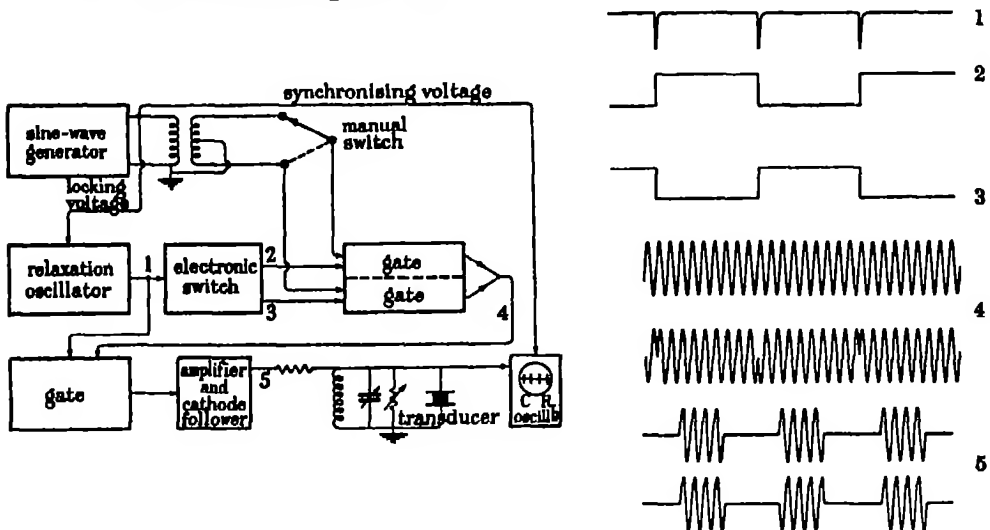
FIGURE 8

The method of generating the transients will be readily understood with the aid of the block schematic diagram (figure 8).

A relaxation oscillator of widely variable frequency determines the overall pulse repetition rate in both stimuli, and since it is locked to a sine-wave generator, the pulse repetition frequency is commensurable with the frequency of the sine-wave generator. If the latter is f, the former is $f/(n+m)$. The relaxation oscillator feeds

a switch which alternately opens and closes two balanced gates leading to a common path. One gate is open when the other is closed and each pulse of the relaxation oscillator reverses the existing state, i.e. the open gate is closed and the closed gate opened until the next pulse. The input to the gates comes from the sine-wave generator through a transformer with a centre-tapped secondary winding from the two halves of which voltages of opposite phase and approximately equal amplitude may be taken . One gate is always fed with one of these voltages, the other gate may be fed with either voltage by way of a manually operated tumbler switch.

The result of the operation so far is that the voltage monitored at the common pathway when the system has been properly balanced has one of the two forms indicated diagrammatically (figure 8, trace 4). When the gates are fed in the same phase the output is a sine wave of constant amplitude with a discontinuity (barely detectable on the monitor tube) occurring at intervals of a fixed number of cycles. When the gates are fed in opposite phase, phase reversal occurs in the output at the same interval apparently instantaneously and without perceptible change of amplitude.

The output of the common path is then applied to a further gate system similar to that used in the experiments of the first subsection. This gate is driven from the relaxation oscillator, the delay in opening and the period for which it remains open being independently controlled. The output of this gate then has the form shown in figure 8, trace 5, when measured across a resistive load. When applied to the Rochelle-salt transducer through a series resistance the pulse was altered in phase and had superposed decremental oscillations at the resonance frequency of the crystal. These effects were eliminated by tuning the crystal with external inductance and capacity to the frequency used and adding enough electrical damping to reduce the Q of the system to a value less than 10. So far as could be seen on the monitor tube with the phase-reversing switch in the off-position every pulse was identical. And in the on-position alternate pulses were reversed in phase exactly and no other changes in the picture occurred.

It was naturally impossible to monitor the resulting wave form in air, since no microphone of the requisite faithfulness and sensitivity exists. It is, however, certain that the air displacement must be that corresponding to the voltage appearing across the crystal, if the crystal is loosely coupled both to the electrical supply and to the air into which it radiates; under these conditions the crystal can be regarded as a monitor instrument, the relation of voltage to displacement being given by the piezo-electric constant. The actual coupling of the crystal to air is certainly small enough, as the ratio of acoustic impedances is about 20,000, and the electrical coupling can, of course, be adjusted to be as low as desired by the use of series resistance large compared with the magnitude of the crystal impedance. Moreover, it can be shown that the overall instrumental accuracy was more than adequate. This was done as follows:

Let the two test stimuli be represented respectively by $AAAAA\ldots$ for the stimulus with all the pulses in phase and $ABABA\ldots$ for the stimulus with alternate pulses reversed in phase. The equipment was set up and adjusted for some value of the parameters such as $f = 5$ kc./sec., $n = m = 10$, for which the audible difference

between the two test stimuli was very marked. It was then left untouched except that the connexions of the transformer at the input to the gate system were varied as follows:

(1) The primary connexions were reversed. The test stimuli were then $BBBBB...$ and $BABAB....$ The difference was as evident to the subject as before.

(2) The input to the gate which normally supplied the unreversed A component was connected to ground. The signals were then in the form $—A—A—$ and $—B—B—$. No difference between these signals could be detected.

(3) A switch was placed in the primary so that its polarity could be reversed with the secondary switch in either position. It was now possible to compare the signals $AAAAA...$ with $BBBBB...$ and also $ABABA...$ with $BABAB....$ Again no difference was detectable to the subject.

These results show conclusively that the electrical symmetry and linearity of the system were adequate and that instrumental inaccuracy made no significant contribution to the difference which the subject detected in the main experiment.

It has been emphasized that for the main experiment the subject was required only to assert that he could or could not detect a difference and was not required to make any judgement of the character of the sound or to say in what the difference consisted. Nevertheless, the subjective character of the sensation is of considerable intrinsic interest, though difficult to describe. There is general agreement that the sound of both stimuli is a very impure 'buzz' in which certain frequencies stand out above a background of neutral pitch.

When the switch from AAA to ABA is made the subjective effect depends on the attitude of the listener. If he has fixed his attention on one of the prominent pitch elements in the buzz, the effect of switching is to cause an apparent rise or fall in pitch It may be either, but a change of pitch is the salient sensation. On the other hand, if the listener is attempting to listen to the buzz as a whole he is conscious not of any change of mean pitch but of a sensation that all the prominences have been reshuffled.

These sensations seem to be completely explicable in terms of what may be assumed to be the distribution of displacement along the basilar membrane. This distribution is calculable if simplifying assumptions are made, but it is far easier and perhaps more convincing to determine it practically by means of a frequency analyzer of selectivity comparable to the ear. This was done by connecting a tunable circuit of Q approximately 200 in series with a high resistance across the crystal transducer as shown, and observing the voltage across this circuit oscillographically.

As the resonant frequency of this tuned circuit is varied continuously, it represents successively each single resonant element of the ear. The voltage across it would be proportional to the *velocity* amplitude of the resonant element at that frequency when the ear is excited by a sound stimulus of the same wave form, if the selectivity of tuned circuit and resonant element were the same, and no allowances were made for undetermined factors such as the variation of match of the ear to air with frequency, etc.

We find that, plotting voltage across the tuned circuit against frequency for the stimuli AAA and ABA, we obtain curves of the kind shown in figure 9. Evidently

the humps correspond to the prominent frequencies detected by the listener. As $(n+m)$ is increased their separation decreases and the distinction between hump and trough becomes less. This agrees with the listener's experience. Reduction of the Q of the tuned circuit reduces the distinction between hump and trough without altering their frequency distribution, and a considerable reduction makes them imperceptible. This, of course, is what would be expected. It is also obvious why a listener concentrating his attention on a particular hump may experience either a rise or a fall of pitch when the stimuli are changed irrespective of whether the change is from AAA to ABA or conversely.
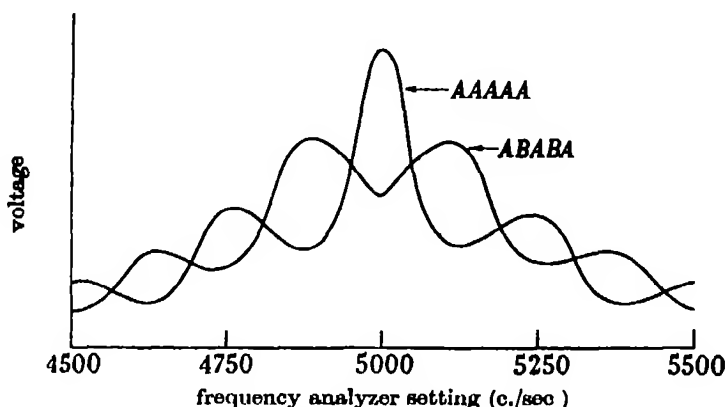
FIGURE 9

For the benefit of anyone repeating these experiments a warning may be opportune. The auditory effect we have been describing is so marked that it is readily demonstrable in an ordinary room against a considerable background of noise. But if this is done it is essential that the transducer shall be closely apposed to the subject's ear. Otherwise a spurious change of intensity will probably be observed in switching from AAA to ABA. This effect is due to interference between a pulse travelling directly to the ear and earlier pulses which have been reflected from the walls, floor, ceiling and furniture. It is critically dependent on the position of ear and crystal and a particular position which gives maximum loudness for AAA will be a minimum for ABA, and the wanted effect will consequently be masked. In order to demonstrate the latter the crystal must be close enough to the ear for signals AAA and ABA to be of equal loudness. Within this range the position of crystal relative to the ear is *not* critical.

3. DISCUSSION　THE INTERPRETATION OF THE ACTION OF THE COCHLEA

Theories

Many different views have at one time or another been held regarding the mode of action of the cochlea. The simplest and rather naïve view that the function of the cochlea was merely confined to that of a microphone (the 'telephone theory') has not withstood the test of time; apart from the inconsistency between the complex

architecture of the cochlea and the supposed simplicity of its function, and apart from the physical difficulties which such an interpretation relegates to the nervous system, the experiments of Galambos & Davis (1943) on single nerve fibres of the 8th nerve are a final disproof of such an hypothesis. They clearly show that the analysis of sound into frequency components has already taken place before the transmission of the information along the nerve comes into play.

Similarly, the theories which sought to combine aspects of this 'telephone theory' with a theory of peripheral analysis now become redundant   According to these it was supposed that the region of excitation in the cochlea was in some way dependent on the incident frequency, but that a more detailed frequency information was conveyed as a correspondence between nerve signals and multiples of the applied waves. Importance was attached to the observed fact that up to frequencies of 2 kc./sec., nerve signals were initiated at every, every other, or every third maximum of the pressure wave. Such a correspondence is, however, the inevitable consequence of the mode of action of nerve endings which detect displacements of the structures on which they are anchored, and which possess the usual refractory and recovery periods. The discovery of partial synchrony between incident sound waves and nerve impulses in the 8th nerve did appear at the time to be a bar to the acceptance of the resonance hypothesis, for, it was argued, if frequency analysis of sound by the basilar membrane is complete, the frequency of impulses in the cochlear nerve fibres should be a function of intensity of sound only. This difficulty, never very real, has also been eliminated by the single nerve-fibre experiments referred to above, which show that partial synchrony is compatible with a consistent relation between intensity of sound and frequency of impulses, provided the latter is estimated over an interval which is not too short. These results also show that the stimulation of a fibre is sufficiently critically dependent on the incident frequency to account fully for the facts of subjective frequency discrimination, so that no further finesses in the transmission mechanism need be envisaged.

Some other and more complex theories are also disposed of by the single nerve-fibre experiments, and in fact we are justified in saying that the clue to frequency is obtained solely from a detection of the pattern of excitation within the cochlea. Many other observations which are now available leave no doubt as to the sense of the change of locus of excitation with frequency, it is generally accepted that the response to high frequencies is centred at the basal end, and that the locus of maximum excitation moves towards the apex as the frequency is lowered. Attempts by different methods to discover the precise positions of the locus for some frequencies have given reasonably consistent results. This aspect of the action of the cochlea, sometimes called the 'place theory', is now virtually undisputed, the aspects on which there is widespread disagreement, however, are the degree of localization of a pure-tone stimulus, and the physical processes contributing to such a localization.

In the interest of clarity we will now distinguish between two aspects of our problem. One aspect is the study of the behaviour of the cochlea, there we shall be concerned with an analysis of the experimental evidence in favour or against some particular description of the movements of relevant points in the mechanism. The

other aspect is the study of the physical processes which are occurring, and which must provide the connexion between cause and effect.

We shall here discuss only the evidence for particular descriptions of the behaviour, and leave the theory of the physical processes to a later paper. With this procedure we shall avoid introducing any bias in the interpretation of experiments; and a bias based on the necessarily incomplete understanding of the physical processes of a biological mechanism should in fact not be allowed to enter into an examination of its behaviour.

An aspect of this behaviour which is of fundamental importance in this study is the rather strict adherence to linearity in the mathematical sense. We can measure the deviations from strict linearity of that part of the mechanism which carries the oscillations by measuring the generation of summation tones and of harmonics as a function of intensity. Whilst such phenomena are of importance to hearing, they can be regarded as a useful imperfection of the mechanism, and can be ignored if we confine our attention to intensities in the vicinity of threshold.

The principle of linearity is of considerable help in describing the behaviour of any device, for it provides a relation between the behaviour in response to pure tones and in response to transients, a relation which is quite independent of the particular physical processes involved. We shall not here go into the subject of Fourier analysis, but merely quote a relevant result, and point out that much misunderstanding in relation to theories of hearing could have been avoided in the past if the implications of this result had been fully recognized. It is there stated that in any linear device the frequency characteristic relating input to output completely specifies the behaviour of this device with respect to any input signal,[*] irrespective of whether this is continuous or transient. Or, expressing this differently, if we know the frequency response curve of any linear device, we can calculate the form of the output which will result from any given input signal, without requiring any knowledge of the processes involved.

From the point of view of behaviour, then, the first question we would like answered by experiments is this. How sharply localized is the response of the basilar membrane to a pure tone? Or, a question which we now see to be related to the former: How quickly does a point on the basilar membrane respond to the sudden onset of a wave, and how quickly does it return to quiescence after the sudden end of a wave?

One might think that the measurements with single nerve fibres would give the most direct evidence, that the frequency response characteristic of a point on the basilar membrane could be identified with that of a single nerve fibre. If that were the case then an estimate of the characteristics of the whole basilar membrane could be synthesized from a knowledge of the behaviour of a number of nerve fibres. It has been argued that this is not justifiable, as the process of nervous detection may be complex, and a single nerve fibre may reflect the behaviour of more than just one point of the basilar membrane. Hence we cannot take these measurements

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[*] Apart from a time delay which is arbitrary, but equal at all frequencies. This, however, is of no relevance in the present context. For a proof of the theorem see Titchmarsh (1937) and Whitehead (1944).

as evidence for the behaviour of the basilar membrane, though we might argue that they will provide circumstantial evidence if their simple interpretation agrees with other results.

Frequency and amplitude discrimination

The most important evidence for the behaviour of the basilar membrane is unfortunately rather indirect. We know the frequency discrimination of which the human ear is capable, and we must suppose that the smallest recognizable increment of frequency changes the pattern of excitation so that at some place a change of amplitude becomes recognizable. We know what constitutes a recognizable change of amplitude in the case of successive signals, as this is a readily measurable quantity, and we might well suppose that this would be similar to the change of amplitude at one place which is required to reveal a change of frequency. The one case is, however, a successive and the other a simultaneous comparison of amplitudes, and it has been suggested that in the latter case the comparison of amplitude may be much more accurate than in the former. Such an assumption would appear to be very unlikely · it would suggest the existence of a mechanism capable of a much finer perception of amplitude than manifests itself in any other way, a mechanism which evolved to its present perfection solely for the purpose of frequency discrimination. We may point out that no detailed mechanism of this sort has ever been described, not even as an example to demonstrate that this is theoretically possible; nor is any other type of sense organ known to furnish an analogy, for it is apparently a general rule that the differential thresholds for successive and simultaneous comparison are of the same order of magnitude   Evidently, for single pure tones a mechanism could exist which would suppress nervous excitation at places away from the maximum, and in this way enhance the definition. But a mechanism of this sort would have to function in the same way with respect to one pure tone, whether or not others are present which excite an overlapping region, i.e it must allow for a linear super-position of frequencies to a fair degree of accuracy. That such requirements are theoretically compatible has not been proved, and appears to us very unlikely.

This is the dividing point of modern opinion regarding the behaviour of the basilar membrane. One view is that the tuning of the basilar membrane is very broad, that any pure tone results in only a flat maximum at one place, and that an accurate comparison mechanism then comes into play, which is responsible for the apparently sharp tuning in evidence at one nerve fibre, and hence for the frequency discrimination actually achieved. The other view, which is the one we hold, and which we consider to be proved by our experiments, is that the behaviour of the basilar membrane itself already shows the criticality to frequency which is imaged in the behaviour of single nerve fibres. We then see that our capacity for frequency discrimination is no more than the natural result of our known amplitude discrimination.

The oscillatory time constants of the cochlea

We will now consider this controversy from the other aspect of behaviour, namely, that of the speed of response and decay   As a general rule we know that the sharper the tuning of the basilar membrane, the longer the time constants which must be

associated with its response to a sudden beginning or end of a wave. These time constants are measurable, as we have shown in the previous section; the values which we find are, as seen in the present context, long (i.e. about 10 msec.).

In the analysis of the experiments we described the results in terms of the value of the parameter Q which a single resonant element must have for its time constant to be the one we find. We then continue to say that the sharpness of tuning of one point of the basilar membrane must be similar to that of a single resonant element of that value of Q.

The mathematical relation between the decay time and the frequency response curve (except in the special case of completely independent elements) is too cumbersome to trace out in detail here. It is, however, clear that the region of the response curve which is of the greatest significance in determining the decay time is in the neighbourhood of the peak. We can hence estimate the actual shape of the frequency-response curve in the neighbourhood of the peak, as we cannot be much in error if we say that its sharpness must be similar to that of a single resonant element of the value of Q specified by the decay time.

Selectivity and frequency discrimination

We may now inquire what value of Q is indicated by earlier measurements of the least detectable increments in intensity and frequency, quantities symbolized conventionally by ΔI and ΔF respectively In table 2 the values for ΔI and ΔF are taken from the work of Riesz (1928) and of Shower & Biddulph (1931). We have selected the values at a reference intensity of 5 db. above threshold since these must lie in the linear region.

TABLE 2

F (c./sec.)	ΔF (c./sec)	ΔI (db.)	lowest value for Q	highest value for Q
63	6·7	7	10	50
125	8	5·5	13	65
250	8	4·5	20	100
500	8	3·6	35	175
1,000	9	3·0	54	270
2,000	13	2 5	65	325
4,000	22	3·0	83	415
8,000	56	4·5	103	515
12,000	90	5·3	108	540

From what has been said above we may expect an increment in frequency at constant intensity to be perceptible only if it results in a change of amplitude at some point on the basilar membrane such as would be produced at constant frequency if the intensity were changed by a just perceptible amount. If ΔI is 3 db., we can write $Q > F/2\Delta F$, and for other values of ΔI a correction factor is readily obtained from the resonance curve. The fourth column of table 2 is obtained in this way and represents a reasonable lower limit for Q. An upper limit is set by the requirements for a smooth variation of sensitivity with frequency. The response curves of two successive resonant elements must overlap so that the difference in their amplitude does not exceed that corresponding to an intensity change of ΔI.

But at the loudness level we have selected there are approximately five rows of hair cells per ΔF and consequently, we assume, five resonant elements per ΔF. Hence we estimate an upper limit for Q five times the lower limit.

As has been emphasized such a calculation is approximate only. Nevertheless, it is significant that the values of Q which we find experimentally lie within the limits assigned, except at 800 cycles, where the experimental value is actually too *low*.

Selectivity and sensitivity

An absolute lower limit for the Q of the resonant elements can also be inferred from the sensitivity of the ear, though this does not seem to have been generally appreciated. It was noted in the introduction that the thermal noise energy in a resonant element corresponds to $2\pi FkT/Q$, and it must be admitted that a threshold stimulus will exceed this power by at least ΔI db. at frequency F and threshold intensity. ΔI at threshold is not a readily assessable quantity, but it is clearly greater than ΔI at 5 db. above threshold which we can quote from Riesz. Hence we can write

$$Q > \frac{2\pi FkT}{P}\frac{X}{Y},$$

where Y is the area over which the ear absorbs sound, X is the factor corresponding to ΔI db. and P is the threshold field intensity in ergs. sec.$^{-1}$ cm.$^{-2}$. If we take F to be 4 kc /sec., ΔI is 3 db, so $X = 2$. P for the average young adult is 2×10^{-10} ergs. sec.$^{-1}$ cm.$^{-2}$ (Sivian & White (1933)); Y is probably not widely different from the area of the tympanic membrane, 0·9 cm.^{3}. Hence $Q > 12$ at 4 kc But according both to Sivian & White and earlier observers (cf Wien), trained and sensitive ears may exceed the average in sensitivity by more than 10 db, so that, for such ears at least, $Q > 120$ at 4 kc./sec. Here also we reach a figure for Q which is in agreement with our results in order of magnitude and inconsistent on any terms with high damping of the resonators of the ear.

Criticism of the evidence for high damping

Our experiments which determine the oscillatory time constants of the inner ear are proof of the existence of comparatively high degrees of resonance. It may be of interest, however, to inquire how the opposing view of nearly critical damping came to be held, and what evidence has been considered to favour it.

Although that view has received considerable attention, and, in fact, may be said to have been the prevailing opinion for two decades, the case for it has never been consistently argued, and we can only find the following arguments which have at one time or another been advanced:

(1) It has been asserted that on purely physical grounds it is impossible for the degree of resonance to be considerable.

(2) It has been thought that the response of the ear to transients and the absence of any subjectively recognizable 'ringing' at the end of a signal indicated a very low degree of resonance.

(3) The evidence of cochlear microphonics was thought to indicate that the generators of the microphonic potential were associated with elements of a low

degree of resonance, and these generators are thought to be the hair cells of Corti's organ.

(4) It has been believed that evidence from auditory psychology, and especially from the phenomena of masking of one tone by another, was consistent only with a very low degree of resonance of the elements of the inner ear.

With the first of those reasons we shall not deal in detail here as it will be treated fully in a further paper by one of us (T.G.). As we have indicated earlier, we are of the opinion that the possible physical limitations should not be allowed to influence an estimate of the behaviour until it is certain that the physical processes are fully understood. We need only point out that if the physical argument is valid for a passive system where the damping is given by the viscosity, etc., of the substances concerned, it is certainly not valid for a system which is 'regenerative' and where the frictional losses can be counterbalanced by a supply of energy from sources other than the acoustic one.

The second reason is clearly fallacious except when applied to the case of very low frequencies. We have seen that the time constant introduced by a degree of resonance Q is given by $Q/\pi f$. No inferences regarding such time constants have led to estimates shorter than 10 msec , which is consistent with the values of Q which we have estimated.

The cochlear microphonic potential

None of the known experiments with the microphonic potential of the cochlea are theoretically capable of leading to an estimate of the degree of resonance of the elements of the inner ear, such estimates as have been advanced can be shown to rest on a misconception.

When a mammalian ear is excited by a noise, then a microphonic potential can be measured by means of electrodes placed near the cochlea. This potential is now thought to arise from the region of the hair cells of Corti's organ, and, it has been argued, that if these were connected with highly resonant structures, then this resonance should produce noticeable distortions in the reproduction of short transients like a 'click' or a sudden reversal of phase of a note (Hallpike, Hartridge & Rawdon-Smith 1937). When it was found that this potential followed the wave form of the applied sound always with great fidelity, then this was thought to indicate that each individual source of this potential followed the sound pattern with the same accuracy, i.e. that each source was very heavily damped.

This argument would be valid only if the received potential had originated from a single resonator; if, as is undoubtedly the case, it is the result of the activity of many elements, then we should have to know the amplitude and phase of each of the contributions in order to analyze the result. A different method of approach is, however, possible As we have mentioned before, the transient response of a device is always completely described by the frequency-response curve, without any reference to the particular mechanism involved. If, then, the frequency-response curve of the microphonic potential is measured, with any particular constellation of the electrodes, then the entire behaviour of the arrangement is known. Any transient response measurement is superfluous, as its result was already fully

contained in the measurement of the frequency-response curve. For any constellation of the electrodes which has been tried such frequency-response measurements have given a very wide band width, as, indeed, would have to be expected on any basis owing to the superposition of the contributions from many sources, with no single source being favoured particularly by the arrangement. The transient response hence shows the high fidelity which it must do, corresponding to the wide band width. We may hence dismiss entirely this evidence adduced in favour of high damping, as by its very nature it could not throw any light on the problem.

Masking

The further evidence which has been cited as favouring the low Q hypothesis is contained in the measurements on masking of one tone by another. It is known that a loud tone will mask others over a wide range of frequencies, and this indicates that the loud tone excites a large part of the basilar membrane. From this it has been inferred that the tuning of the basilar membrane is flat, i.e. that it is heavily damped.

If we attempt to investigate this point quantitatively on the basis of published figures for the masking effect, there are certain inherent difficulties. The degree of masking is defined by the extent to which the threshold for a test pure tone is raised when sounded simultaneously with a masking tone of chosen frequency and loudness level. This rise in threshold is attributable to the oscillation produced in the elements resonant to the test tone by the masking tone. In order for this test tone to be perceived in the presence of the masking tone the amplitude of oscillation must be further raised by an amount corresponding to ΔI *at this level*, i.e. by an increment which, expressed as a percentage of the initial amplitude, may be nearly independent of the masking intensity but which in absolute units of intensity will be nearly proportional to it. The efficacy of one tone in masking another is thus a consequence of the approximately logarithmic intensity characteristic of the ear.

At first sight it would appear that the resonance curve of an element at a particular frequency could be estimated by measuring the threshold to a tone of that frequency in the presence of a succession of masking tones of different frequency and all of the same sensation level of intensity. But, apart from the uncertainty of the physical meaning of 'sensation level' in this context, there are two other sources of difficulty. First, when the test and masking tones are close in frequency, beats reduce the masking effect giving the characteristic double hump to the masking curve. This effect makes any inference about the resonance curve from the masking curve at low-masking intensities quite uncertain. Secondly, at high-masking intensities, masking is largely attributable to intra-aural harmonics of the masking tone when the latter is lower in frequency than the test tone, and this prevents any certain deductions about the resonance curve on the low-frequency side of resonance. Nevertheless, it is clear (see figure 10b) that with loud masking tones of higher frequency than the test tone the masking effect falls very steeply as the separation in frequency between masking and test tone increases. And this in itself is indisputable evidence of high selectivity.

It is instructive to compare a typical masking curve with the frequency-response curve of a single fibre preparation (Galambos & Davis 1943). When allowance is made

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for the absence in the latter of the double hump referred to above there is an obvious familiar resemblance between the two. Both show an extremely steep slope on one side and evidence of the effect of intra-aural harmonics on the other. (Both curves are indicative of a steeply falling response on the *high*-frequency side of the resonance curve of a resonant element. The apparent difference is due only to the way in which the results are expressed, see legend to figure 10.)
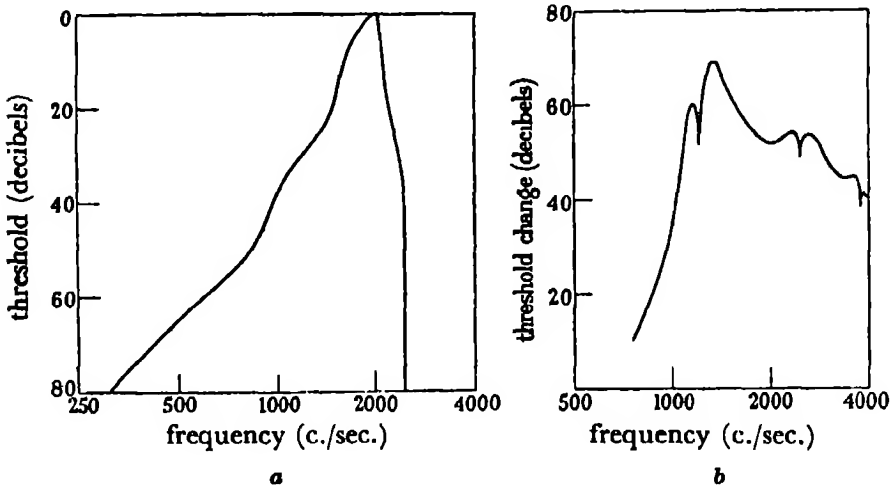
FIGURE 10. *a* (after Galambos & Davis). The response curve of a single fibre preparation most sensitive at 2 kc./sec., showing the intensity required to excite it at other frequencies. The intensity rises very steeply as the exciting frequency is raised above the frequency of maximum sensitivity. *b* (after Wegel & Lane). The intensity which a test tone must have at the frequency indicated in order to be heard in the presence of a masking tone of 1200 c./sec. 80 db. above threshold. The threshold intensity for the test tone rises very steeply as the frequency of the test tone approaches that of the masking tone from below. As it must be assumed that the resonance curves of neighbouring elements are similar, this curve must be nearly the mirror image of that which would be obtained if the frequency of the test tone were kept constant and the frequency of the masking tone were varied. The analogy with *a* is then obvious.

The steepness of slope is in fact too great to be consistent with any probable value for Q if the resonators are assumed to be independent. It is an indication that in addition to their high selectivity there must also be some degree of coupling between neighbouring elements. This point will be dealt with further in a later paper. But it may be as well to point out here that it is not possible with any assumed coupling factor to make either the masking results or the single-fibre response curves consistent with a low value of Q in the neighbourhood of 2 kc./sec.

We have been unable to find that any other pieces of evidence have been taken seriously as supporting the hypothesis of high damping. These, as we have shown, are in fact either neutral or valid evidence for the contrary view, namely, that the selectivity is very high. All the experimental evidence available seems on this view to be self-consistent. And in conclusion it may be useful to summarize some of the data in a diagrammatic form.

Conclusion

It was stated in the introduction that the human ear operates as a frequency analyzer for the purpose of coding the information of a train of sound waves into a form which the central nervous system can accept. For pressure variations whose period is short compared with the time constant of the nervous system, the use of an analyzer of this kind is the only way of coding which does not necessarily involve loss of information; and for such pressure variations the ideal system of coding, from this point of view, would be achieved by resonators whose time constant was in a particular relation to the speed of action of the nervous system. An 'ideal' ear would hence be one where all resonators possessed the optimum time constant. The degree of resonance (Q) would then be proportional to the resonant frequency of each element; and the number of elements required in any given interval of frequency would then only depend on the degree of smoothness with which the interval is to be covered. The required degree of smoothness, we suggest, is such that the available intensity discrimination is just insufficient to reveal the difference of excitation of neighbouring elements. If we stipulate that for the 'ideal' ear the intensity discrimination ΔI is constant over the frequency band, then the required number of resonators in any octave is proportional to the frequency. The number of discriminable steps (ΔF) of frequency per octave would then also be proportional to frequency, which is a necessary condition for avoiding a loss of information.

For pressure variations whose period is the same or longer than the limit set by the nervous system it is theoretically possible to obtain all the available information either by frequency analysis or by time discrimination or by a compromise, but evidently these alternatives will not be equally convenient, and in the mammal frequency analysis is preferred to time discrimination. This implies that the oscillatory time constant *and* the time constant associated with the discrimination of amplitude must increase for the lower frequencies and the selectivity must tend to a constant value with diminishing frequency. If it were otherwise, the nervous system could and would respond to instantaneous amplitude of oscillation rather than to the envelope of peak amplitude, i.e. frequency discrimination would be sacrificed to time discrimination, which would involve the use of two different systems of recognition in the central nervous system

Referring now to figure 11, it will be convenient to consider the lower and upper halves of the auditory spectrum separately. Below 1 kc/sec. the ear conforms closely to the ideal. The number of resonators per octave and the number of discriminable intervals of pitch per octave are very nearly proportional to frequency, and the time constant derived from two sources (see legend to figure) also corresponds reasonably to expectation. The increase in time constant at the lower frequencies (while the number of resonators per octave is still diminishing as the frequency diminishes) means that the overlap of the resonance curves of neighbouring elements is less at the lower frequencies. We might therefore be led to expect the increased ΔI which, in fact, we find.

The close correspondence with the ideal behaviour which the ear here displays is consistent with the now established fact of acoustical engineering that in com-

munication systems no appreciable economy of band width is possible in this range of frequencies without loss of information (Gabor 1946).

In the upper half of the spectrum the state of affairs is very different; the number of resonators and the number of discriminable intervals of pitch per octave are in
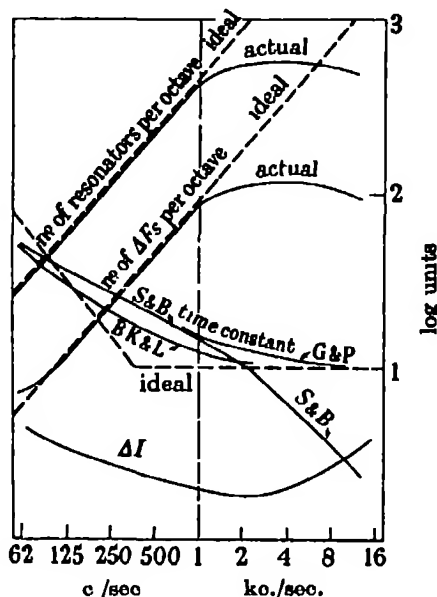
FIGURE 11. Dotted lines represent the parameters of an ideal analyzer. Solid lines represent inferences from experimental data for the human ear. Since the parameters are mutually dependent, the *position* of the dotted lines is to some extent arbitrary and has been chosen to give the best fit. The slope is determined by the considerations enumerated in the text.

1. *Number of resonators per octave.* The solid line is computed from the data of Culler (1935), Stevens, Davis & Lurie (1935) and Crowe, Guild & Polvogt (1934) on the spatial distribution of loci of maximal excitation in the cochlea, on the supposition (1) that there are 2500 equally spaced hair cells of the inner row in the human organ of Corti and (2) that each such hair cell corresponds in position to a single resonant element. For the higher frequencies evidence for the human ear is available. For the lower frequencies the solid line represents an inference from experiments on other mammals.

2. *Number of ΔF's per octave.* The solid line is computed from the frequency discrimination data of Shower & Biddulph (1931) at the level 5 db. above threshold. At higher intensities it would be shifted upwards approaching but not reaching the first curve, and non-linear effects would reduce the correspondence with the ideal.

3. *Time constant* (expressed in msec.). The dotted line corresponds to a time constant equal to 10 msec. or four periods, whichever is longer (see text for explanation of the increase at low frequencies). The solid line (S. & B.) represents the lower limit for the time constant which is necessary to explain the data on frequency discrimination. It is computed from table 2 column 4. The solid line (B., K. & L.) is derived from measurements by Bürck, Kotowski & Lichte (1935) of (1) the time required for the appreciation of pitch, (2) the least perceptible interval in the onset of two tones. The two methods give identical results in this range. The solid line (G. & P.) represents our own (smoothed) results. Note that the experiments of B., K. & L. were performed at a higher level of intensity than those quoted from B. & S., and G. & P., and therefore probably appear too low in this figure.

4. ΔI. The solid line represents the least perceptible increment of intensity at the level of 5 db. above threshold according to Riesz (1928). ΔI is expressed in bels to avoid superimposing the curve on the curves for the time constant. At higher intensity levels this curve would be shifted downwards, and the rise in ΔI at the extremes would be less marked.

this range nearly invariant with frequency, as is also the time constant computed from our own experimental results. The lower limit for the time constant required to explain the observed frequency discrimination nevertheless falls steeply with increasing frequency. This means that in this region the selectivity of the resonators is much higher than would be necessary for frequency discrimination; and here also the rise in ΔI, which is to be expected as a consequence, occurs. The high degree of resonance must hence be attributed solely to the need for sensitivity in this region.

The departure from ideal behaviour at the higher frequencies is clearly not due to their unimportance, for more than two-thirds of Corti's organ and of the cochlear nerve fibres are concerned with frequencies above 1 kc./sec The falling off in performance must be regarded as enforced by economy. It is, indeed, easily seen that to deal with frequencies up to 16 kc./sec. as lavishly as up to 1 kc./sec. would require a cochlea at least three times as large as the one we have.

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Hearing.   II. The physical basis of the action of the cochlea

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An attempt is made to discover the physical processes in the cochlea which would yield results in agreement with observations It is shown that the assumption of a 'passive' cochlea, where elements are brought into mechanical oscillation solely by means of the incident sound, is not tenable.

The degree of resonance of the elements of the cochlea can be measured, and the results are not compatible with the very heavy damping which must arise from the viscosity of the liquid For this reason the 'regeneration hypothesis' is put forward, and it is suggested that an electromechanical action takes place whereby a supply of electrical energy is employed to counteract the damping.

The circumstantial evidence for such a process is considered, and it appears that the cochlea microphonic potential, hitherto an unexplained by-product of the action, forms an important link in the chain of events Some implications of the theory are discussed, and ways of testing it are suggested.

1. INTRODUCTION

In the previous paper, part I (Gold & Pumphrey 1948), there were set out certain conclusions regarding the behaviour of the cochlea, which were necessary to a consistent interpretation of available experimental results. It was shown that the degree of resonance of each receiving element of the basilar membrane must be high, and that the opposite view of nearly critical damping was not tenable. The values of the degree of resonance Q which we found ranged from 60 to 250 in the frequency range between 1 and 10 kc./sec.

In that paper the objections which have been advanced against the assumption of a high degree of resonance were considered; it was shown that all objections based on experimental results could be dismissed, but it was left to this paper to discuss the objections based on physical considerations, namely, on the viscous damping of the basilar membrane.

2. THE VISCOUS DAMPING OF THE BASILAR MEMBRANE

The basilar membrane is entirely immersed in liquid, and this fact, together with the known size and density, enables an estimate to be made of the least amount of viscous damping which may be present. The treatment by Stokes (1901) of vibrating strings in a liquid is perhaps the most suitable for obtaining an upper limit of the value of Q. We do not wish to imply that the basilar membrane ought to be regarded merely as a series of independent strings for the purpose of an accurate calculation; but for our purposes it will suffice to show what degree of resonance could be achieved by such strings. We might equally well take some different geometrical constellation, and, so long as we take similar characteristic dimensions and densities, we would not obtain a very different upper limit to the value of Q.

The following table gives the maximum values of Q which a string of density 1 and diameter 0·1 mm. could have when immersed in water:

resonant frequency (c./sec.)	Q
36	1·2
125	1 8
500	3·3
1,100	5
2,000	6
20,000	20

The value chosen for the diameter of the string is certainly generous. Smaller values would make Q lower still, but with the value chosen the possibility of larger sections of the membrane oscillating as one unit is covered.

We see that the values of Q of such a mechanism would be much too low to account for the observations. The values determined for the human ear were between 15 and 25 times as high, and if, for instance, we wished to account for that by a lower viscosity of the liquid, then we should have to suppose that it is 200 to 600 times less viscous than water. This, of course, would be quite unrealistic.

We therefore have to conclude that the physical argument regarding the degree of resonance in the cochlea is valid, if the cochlea is regarded as a passive device; no constellations of resonances of sufficiently high values of Q are possible within such a small liquid-filled space. Even if every uncertain or inaccurately known quantity, including the experimental values of Q, were given the full benefit of the doubt, we should still be very far from reaching any agreement between the observations and the physical theory.

It is with this difficulty in mind that we discard the idea of a passive oscillatory mechanism in the cochlea, and put forward the 'regeneration hypothesis' As a more complex theory it can be justified only by the absence of a simpler one which is in agreement with physical principles and experimental results.

3. THE REGENERATION HYPOTHESIS

In considering the oscillations of single fibres of the basilar membrane we found that viscosity introduced a resistive term which was too great. If another term existed which was of similar magnitude and opposite sign, then oscillations of 'high Q' again become possible Such a term expresses a supply of energy from another source (negative resistance). The behaviour of a resonator under the influence of a 'positive' and a 'negative' resistance would in all respects be similar to one under the influence of one resistance, of a value equal to the sum of the two, provided that sum is positive.

This principle is widely recognized in radio engineering, and receivers employing it are called 'regenerative receivers'. They are used frequently when resistive losses would otherwise make their frequency selectivity too poor. In their design a certain fraction of the output voltage is again applied to the input. If the voltage-amplification factor is n, then it is a necessary condition that the fraction which is returned

is less than $1/n$ of the output voltage. A greater amount of 'positive feedback' would result in a self-oscillatory system. A signal, however small, which is present at the input would cause a larger one to be applied after one amplification, and this process would repeat. As the feedback fraction is varied between zero and $1/n$, the band width of the receiver will change from its value, in the absence of feedback, to zero. By the correct choice of the feedback fraction any desired selectivity can be obtained. In this case an external source of power is required, not necessarily to obtain an output of greater power than that contained in the input, but in order to make the output a sufficiently critical function of frequency. The same object would be achieved by a filter network, provided that components existed which were free from resistive losses to the desired degree. Our problem is so similar to that of the radio engineer who cannot obtain sufficiently loss-free components, that we may well search for a solution similar to his

Before we describe the mechanism by which, we suggest, such regeneration is brought about, we will describe two electromechanical transducer actions which have been widely investigated.

(a) The cochlear microphonic effect

In all mammalian ears that have been investigated, an effect known as the cochlear microphonic effect has been found (Davis, Derbyshire, Lurie & Saul 1934) Sound waves applied to a 'live' cochlea have been found to result in an oscillatory electrical potential of sufficient intensity to be detected by a sensitive instrument outside the cochlea This oscillatory potential is, so far as one can tell, always synchronous with the applied sound. Its functional relation to the other electrical effect, the nervous impulses, is not known, but it is now certain that it plays no direct part in nervous transmission. This effect has been explained as a curiously large by-product either of the distortion of the basilar or of Reissner's membrane, or of the action of nerve endings and their associated hair cells. Various attempts have been made to judge whether the energy contained in this electric field was small enough to be derived directly from the sound, such attempts cannot, however, give a conclusive result, so long as no estimate can be made of the size and separation of the surfaces across which the voltage appears, that is, without a knowledge of the capacity, and without a knowledge of the screening properties of the intervening material. Such calculations, however, make it appear rather unlikely that the effect could be so great if it were merely due to a conversion of energy, and this favours the view that the microphonic potential represents a modulation by the sound waves of a supply of energy from other sources.

(b) The audibility of electrical signals

The reverse of the cochlea microphonic effect can also be observed (Gersuni & Volokhov 1936). A large oscillatory electric field applied from outside the cochlea results in a sensation of hearing. The noise which is so heard is of the same frequency as the applied electric field, and hence it cannot be explained as a direct electrical interference stimulating the nerves; such an effect can also be brought about, and

in that case the noise heard covers a large part of the frequency band and is un-correlated with the applied frequency. For the correct frequency to be heard, the electric field must have caused mechanical action, which would then in the normal way be analysed for frequency. (Any purely electrical frequency analysis in the cochlea cannot be contemplated.) Again it is not possible to judge the conversion efficiency of the process, but in this case the energy contained in the applied electric field is certainly more than adequate to produce the effect by a direct conversion of energy of the same type as a piezo-electric crystal would achieve

These two phenomena might be explained as the result of one reversible transducer action, but no evidence exists to support this view. Let us assume that they result from different causes, more specifically that the cochlea microphonic effect results from a modulation of a further source of energy such as could be supplied by some form of electrochemical action. The acoustic energy which would then be required to modulate an electric current could theoretically be arbitrarily small As to the second phenomenon, that of 'hearing an electric field', no specific assumption is required.

We now see that, with this one assumption about the nature of the 'microphonic effect', we would have a feedback channel. An oscillating fibre of the basilar membrane would produce an electric field. This would, in turn, by means of the second transducer action, result in a mechanical force impressed on the fibre.

A priori we can say nothing about the magnitude of this feedback. All we can say is that the microphonic electric field may contain a greater amount of energy than that dissipated by the oscillating fibre, and that this may be reconverted into mechanical energy with an efficiency close to unity. Neither action would introduce any physical difficulties, nor, indeed, would they be actions of a very different character from many that have been investigated in physiology.

We would then regard the cochlea no longer as a passive instrument where nerve endings merely record the displacement due to an applied force, but as an active mechanism where an applied signal releases a chain of events involving an additional supply of energy.

Now let us consider the implications of this hypothesis. First, and most im-portant of course, the feedback action may largely cancel the resistive losses and would thereby enable the fibres to oscillate as 'high Q' elements. For this purpose the phase of the signal fed back would have to be in a particular relation to the primary oscillation; this is best seen by considering the forces which are operative. The differential equation of a natural oscillation in the presence of liquid damping is

$$M \frac{d^2x}{dt^2} = -kx - c \frac{dx}{dt},$$

where $-c(dx/dt)$ is the force introduced by viscosity. The regeneration process will add a further term to the right-hand side. If this term is to have no other effects than to decrease the damping, then it must be of the form $+r(dx/dt)$. The feedback force would hence have to be proportional to the velocity of the fibre, and in phase with it.

If those conditions were satisfied, then the natural oscillations of a fibre would have the same properties as a passive system in the presence of a smaller amount of liquid damping.

The physical requirements for such an action are, in principle, quite simple. The microphonic potential generated at one element must be proportional to the displacement velocity of that element, a function which a simple physical device could fulfil, and for which no further tuning of an electrical or mechanical nature need be envisaged   Our knowledge of the cochlea is unfortunately not adequate to make any definite suggestions regarding the parts of the structure which are concerned in this process. As a first guess we may look to a region where the velocity amplitudes are greatest, and this would appear to be the neighbourhood of the top of Corti's arches and the lower side of the tectorial membrane; it is there that any vertical oscillation of a part of the basilar membrane must produce a large flow in and out of the cavity formed by those two structures.

The second part of the action, the reconversion of the electric field into mechanical action, should then be a process where the displacement is proportional to the voltage, and in phase with it. This would be quite analogous to a piezo-electric process. The locus of this action might again be thought to be in the immediate vicinity of the former, the hairlets spanning the gap between Corti's arches and the tectorial membrane are in a favourable position, and those, or some of those, might well be concerned.

Perhaps we should not attach much weight to this guesswork regarding the structures involved in a regenerative action, it is of interest, however, that no argument denying the existence of regeneration can be based on an inadequacy or an absence of suitably placed structures.

The magnitude of the feedback effect we require for a satisfactory explanation of experiments is, of course, so large as to come precariously close to cancelling the resistive losses. At a frequency of 2 kc./sec. a Q of 6 would be possible without feedback. If the actual Q is 160, then the feedback power should be some 96 % of the power lost through viscosity, or, expressed differently, 24 % of the stored energy of an oscillating fibre should be supplied per cycle. If the feedback ever exceeded the losses, then a resonant element would become self-oscillatory, and oscillations would build up a level where linearity was not preserved. The final amplitude which such oscillations would reach would then be that where the losses are again equal to the feedback power. A permanent adjustment of that accuracy would seem to be impossible when we consider the unsteadiness of the framework, and some form of self-regulating device would have to exist.

'*Ringing' of the ear.* In spite of such a self-regulating mechanism we might expect that occasional disturbances would bring an element into the region of self-oscillation, when it is normally so close to this condition. If this occurred, then we should hear a clear note which would persist until the adjusting mechanism has regained control, or until the nervous sensitivity has decreased sufficiently. It is very tempting to suggest that the common phenomenon of 'ringing of the ear' is frequently of this origin, and not always a central nervous disturbance. The phenomenon referred to here is the sensation of a single clear note of steady pitch,

corresponding to a steady stimulus of one or at any rate a very small number of nerve fibres. The importance of this 'ringing' phenomenon in this connexion does not lie so much in the direct additional evidence it might present for this theory, but in the fact that it may provide the basis for a crucial experiment. If the ringing is due to actual mechanical oscillation in the ear, then we should expect a certain fraction of the acoustic energy to be radiated out. A sensitive instrument may be able to pick up these oscillations and so prove their mechanical origin. This would be almost a conclusive proof of this theory, as such a generation of sound on any other basis is exceedingly unlikely.

When we attempted this experiment we did not succeed in making our ears ring with the required persistence. Further, an estimate of the subjective intensity indicated that the sound energy available outside would only barely exceed the noise level of a sensitive amplifier. A specially designed microphone and amplifier, previously tuned to the correct frequency, might be of sufficient sensitivity Another approach may be to search for pathological cases where 'ringing' of this nature persists at a higher intensity.

There is, however, another way of ascertaining whether ringing is of mechanical origin. If an external source of sound is arranged to supply a note very close in frequency to the 'ringing' one, then we should hear beats if, and only if, a mechanical ringing were present. If the ringing were of nervous origin, then at a high frequency no addition with respect to phase with the external tone would be expected (There is an effect of 'binaural beats' which depends on phase information conveyed by the nerves for the beating to occur in the brain, but this only happens at low frequencies (below 700 c./sec.).)

The phase of the regenerative force. In considering the effects of regeneration we have so far confined our attention to that component of a feedback force which is in phase with the velocity amplitude of an element There are no experimental data regarding the phase relation of the two transducer actions (the 'microphonics' and the 'hearing of electrical signals'), and hence we have no knowledge of the phase of the feedback action.

If a component of force existed which was in quadrature with the one we have considered, then it would be in phase with the elastic restoring force. The effective restoring force, and hence the resonant frequency, would then be altered, and so the tuning of an element may be substantially different from the purely mechanical one. If this were the case, then any lack of amplitude linearity, which has to be expected at high intensities, would show up as a change of tuning It may be that the well-known puzzle of the apparent change of pitch with intensity falls into the province of these considerations.

4. CONCLUSION

In the attempt to interpret on a physical basis the action of a biological mechanism we are generally forced to make many assumptions. For our own convenience we postulate a simple action, until experimental evidence shows a more complex one to be present. But there is no virtue other than convenience in this procedure, for biological mechanisms do not appear to have any preference for simple rather than

obscure or complex physical principles. To postulate the absence of a complicated process is frequently as uncertain an assumption as to postulate its presence.

Previous attempts to interpret the action of the cochlea have led to apparently unavoidable inconsistencies. The degree of resonance of each element required to explain the performance was quite different from that which could be explained by postulating a passive mechanism. But to postulate passivity was no more than a convenient assumption (though this was not recognized) When we fail to account for the observations with such an assumption then we have to discard it; and some sort of regenerative process is the only alternative.

Finally, we have to consider the problem from the point of view of evolutionary advantage. As has been pointed out in the previous paper, a high degree of resonance is of advantage with respect to sensitivity, and a process of regeneration is hence of advantage in so far as it is required to achieve such a resonance. But there is a further advantage:

A nerve ending which is to act as detector will require to absorb some energy before responding, though this may be very little, a limit is set by its own thermal agitation which should in general not reach the triggering intensity. The inter-position of a feedback stage (a linear amplifying device, and hence not possessing any threshold) makes a construction possible where the nerve ending abstracts much energy from the mechanical resonator, i.e. loads it heavily. This loading will be similar to the viscous damping which we discussed, and it can hence be counteracted in the same way. The sensitivity would no longer be limited by the thermal agitation of the nerve fibre, but by that of the resonator, and as the latter is a tuned element and the former is not, the two limits will be substantially different. (This is a principle which is sufficiently well known in radio engineering for a detailed treatment here to be unnecessary.)

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Studies on the genetic and antigenic basis of tumour transplantation

Linkage between a histocompatibility gene and 'fused' in mice

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Inoculation of mice from appropriate crosses with two strain A tumours and one dba (subline 2) tumour showed close linkage of such a gene with the locus for 'fused'

With the A strain it was shown that the gene was identical with that for antigen II present in the erythrocytes The gene is therefore labelled H_2

Of sixty-nine backcross mice tested, both serologically and by tumour inoculation, sixty-five gave concordant results, four discordant results. In general, it appears that serological tests are preferable to tumour inoculation in the identification of genotypes

On serological grounds it is suggested that the dba gene is an allele of the above and should be called H_2^d. It is possible that there is a long series of alleles at this locus

The indicated genotype of the strains used is as follows: A, $H_2 H_2$, *dba*, $H_2^d H_2^d$, CBA, C 57 and P, $h_2 h_2$; CA, $Fu h_2//fu h_2$ Further tests may show differences in the h_2 allele in the last four strains.

It is uncertain whether any cross-overs occurred in 257 backcross mice tested

INTRODUCTION

The genetical laws governing tissue transplantation are accepted with but minor reservations by all workers in the field, susceptibility being determined by a complex of dominant genes. The number of factors involved varies with the hybrids studied and with the tissues employed. The number required by a tumour is likely to diminish on continued transplantation (see reviews by Bittner (1935) and Little (1947)).

In a recent theoretical study Snell (1948) has suggested that the genes concerned should be called histocompatibility genes, denoted by the symbols H or h according to the usual convention in genetics. Previously, Gorer (1937b, 1938) had put forward the hypothesis that these genes were identical with those determining iso-antigenic differences.

Loose genetic linkage between a histocompatibility gene and 'dilution' was found by Bittner (1933), whilst sex linkage was found by Strong (1929), both of whom used tumour inoculation as an indicator. Sawin, Griffith & Steward (1944) found linkage between the A antigen and brachy, a form of dwarfism in rabbits, the genes showing $36·8 \pm 3·2 \%$ crossing-over. In the present investigation both tumour transplantation and haemagglutination were used in the study of linkage.

The study was initiated by Snell and his colleagues, who found linkage between susceptibility to tumours from strains A and dilute brown (*dba*) and the locus for 'fused', the latter being a deformity of the tail of varying extent. In extreme cases

the tail is reduced to a thickened twisted rudiment, however, a variable number of individuals carrying the gene may have normal tails. Many intermediate degrees of the deformity may occur. Gorer had used *A* strain tumours in his former studies (1937*b*, 1938 and 1942) and found that the pertinent antigens were shared by the erythrocytes and fixed tissues, it being fairly simple to obtain strong iso-agglutinating sera. One antigen, denoted antigen II, was found to be particularly potent in the stimulation of antibodies and as a histocompatibility gene. The *dba* strain had not been studied serologically; certain results will be reported below.

MATERIALS AND METHODS

Six inbred strains of mice were used. Four of these, strains *A*, *CBA*, *C*57 black and *dba* (subline 2) are well known. The fused gene was carried in heterozygous form (the viability of the homozygote is poor) in the *CA* strain, the genetic formula of which is $Ca\,Ca\,.\,Fu\,fu\,.\,Ww$. The *P* strain has been built up as a multiple recessive strain with the constitution $aa\,bb\,d\,se/d\,se\,pp\,rr$. The *CA* strain is therefore homozygous for caracul (giving a wavy coat), heterozygous for dominant white (giving a pied coat) as well as for fused, whilst the *P* strain is non-agouti, pink-eyed, dilute brown, with short ears and rodless retinae.

Two *A*-strain tumours were used. One of these (15091*a*) is of mammary origin, opinions may differ as to whether it is a spindle-celled carcinoma or a sarcoma. It is highly pleomorphic with numerous giant cells. The other (*C*1300) has been diagnosed as a neuro-blastoma. In both cases if F_1 hybrids between the *A* strain and a resistant strain are mated to resistant mice, about 50 % of the offspring will succumb. This result indicates that a single gene is involved. Although 15091*a* is the more virulent of the two, it will be convenient to pool the results of both.

The *dba* tumour (*P*1534) is a lymphogenous leukaemia.

The crosses that can be used advantageously to detect linkage between histocompatibility genes and known loci have been discussed by Snell (1948). The one with which we are concerned here consists of a cross of *A* or *dba* strain mice to fused *Ca* strain individuals. The F_1's were crossed to *C*57, *CBA* or *P* mice, and their offspring tested for erythrocyte antigens and/or inoculated with the appropriate *A* or *dba* tumour. In the later tests, fused mice from first or second backcross of the F_1 to the *A* strain were used for the cross to the *P* strain or the non-fused *CA* mice. Assuming that the *A* strain carried a gene H_2 for tumour susceptibility, linked with *fu*, and that the *C*57 black strain carried the corresponding allele for resistance, the final cross where these stocks were used was $F_1(H_2\,fu/h_2\,Fu) \times C\,57(h_2\,fu/h_2\,fu)$ and hence genetically a backcross. The same applies to crosses involving the other strains.

To produce antisera, *C*57 black mice were inoculated subcutaneously with 15091*a*, *C*1300, or *P*1534. The inoculation was usually repeated three or four times, and the mice bled about 10 days after the last inoculation.

The technique of bleeding and performing the agglutination tests has been described elsewhere (Gorer 1938, 1948). Pooled sera from about six *C*57 blacks were used. All linkage data were obtained with anti-15091*a* for reasons given below. The

sera must be absolutely fresh, as partial antibodies are formed rapidly on storage (Gorer 1947).

Tumour inoculation was by the trocar and cannula technique.

RESULTS

(1) *With A strain tumours*

When performing serological tests with hybrids there are three sources of error in classification. Heterozygotes give weaker reactions than homozygotes, sometimes to a degree that makes classification very difficult. Secondly, iso-agglutinating sera may show a pro-zone, which is likely to be considerably exaggerated in heterozygotes. Lastly, their sera may contain more than one antibody (Gorer 1938, 1947). Both of the two last are more marked with anti-C1300, indeed, in one case it was found that all of twelve hybrids showed strong agglutination. However, as can be seen from table 1, strict precautions must be taken with anti-15091a. At one time complete titrations were done with both the pure strain and hybrids as shown in the table. This is unnecessarily laborious; it is sufficient to do a rapid titration against the A strain and use two or three dilutions that give optimal results for the hybrids. In order to guard against the presence of more than one agglutinin, or to confirm cases of very weak agglutination, an absorption test was done. The serum was suitably diluted (usually about 1/20) and 0·2 ml. added to the red cells remaining after the suspensions had been made. Following about 30 min. incubation the sera were tested against A cells. As can be seen from table 1, in spite of inevitable variations in the volume of cells, the results are beautifully clear-cut. In only a few instances was it necessary to repeat the experiment.

TABLE 1. A COMPARISON OF HYBRID AND A STRAIN RED
CELLS TO ISO-AGGLUTININS (ANTI-15091a)

	serum diluted one in							
number of mouse	2	4	8	16	32	64	128	256
A strain	+	+ +	C	C	C	C	C	+ +
backcross $(Ca \times A) F_1 \times P$32	–	–	–	–	–	–	–	–
backcross $(Ca \times A) F_1 \times P$152	–	+	+ + +	a.c.	C	a.c.	±	–
backcross $(Ca \times A) F_1 \times P$158	–	–	+ +	+	±	±	·±	tr.
backcross $(Ca \times A) F_1 \times P$103	+ +	+ +	tr.	–	–	–	–	–

	serum diluted 1/20 absorbed with cells of				
	A	32	152	158	103
result of test on A cells	–	C	–	–	C

C = complete agglutination. a.c. = almost complete, etc.

A further point to be considered was whether the pertinent antigen was antigen II. This was tested by comparison with the CBA strain, which shares antigen I with the A strain but differs from it with regard to II (Gorer 1936; see also p. 505). Serologically the antigen appeared to be II, and this was confirmed in a small backcross to the CBA strain. Had antigen I been concerned all the animals would have been positive, in fact five were positive and three negative.

TABLE 2. TEST FOR LINKAGE WITH ANTIGEN II AND FUSED

| phenotype of mice | number of mice with | | |
	antigen present	antigen absent	total
fused	0	37	37
normal	37	5	42
totals	37	42	79

Table 2 shows the pooled results for all crosses segregating for fused and antigen II. It is clear that the two loci are closely linked. There are no certain examples of crossovers. The five animals with normal tails but lacking the antigen may have carried the gene for fused (see Reed 1937). Genetic tests of these animals might have settled the matter but for the fire at the Jackson laboratory.

TABLE 3. THE INFLUENCE OF ANTIGEN II ON TUMOUR INOCULATION

| | response to tumour | | |
	+	—	total
antigen II present	28	3	31
antigen II absent	1	37	38
totals	29	40	69

Table 3 shows the results obtained with animals tested for their response to tumour inoculation and for the presence of antigen II. In general, the results agree well with those obtained previously (Gorer 1937b, 1942). The three animals that were resistant but lacked the antigen will be discussed later. Taking all the data together it seems justifiable to refer to the gene concerned as H_2.

TABLE 4. LINKAGE OF H_2 TO FUSED AS SHOWN BY TUMOUR INOCULATION

| phenotype of mice | response to tumour | | |
	+	—	total
fused	1	75	76
normal	77	24	101
totals	78	99	177

Table 4 shows the segregation of fused and tumour susceptibility where the original cross was $A \times CA$, regardless of serological testing. Again linkage is clearly demonstrated. Although the $F_1(A \times CA)$, or fused mice from a backcross to A, were mated to four different strains (non-fused CA, CBa, $C57$ black and P), one-factor ratios and linkage with fused were obtained in each case. This accords with the fact that serological tests of these strains show absence of antigen II. Genetically, these are therefore $h_2 h_2$, though the possibility remains that they carry slightly different alleles.

The one fused individual that succumbed died at 6 weeks following inoculation with 15091a. Of the non-fused susceptible mice, thirty-three were inoculated with 15091a rather than C 1300. All of these died at 5 weeks or earlier. This suggests that

the one fused positive mouse was genetically resistant and hence a non-cross-over. Some or all of the non-fused mice were probably genetically fused (Reed 1937), so that they also fail to prove the occurrence of crossing-over between Fu and H_2. All of the 177 mice included in table 4, with a single exception, were from matings in which the male was the heterozygous parent ($FU\,fu$). Had the female been the heterozygous parent the percentage of overlaps would have been higher (Reed 1937, Gruneberg 1943, p. 191).

TABLE 5. THE SEGREGATION OF FUSED IN VARIOUS BACKCROSSES

tumour-susceptible grandparent	sex of F_1	fused	normal
A	♂	77	104
dba	♂	26	17
dba	♀	12	21

TABLE 6. LINKAGE OF $H_2{}^d$ TO FUSED, TESTED WITH *dba* TUMOUR

	response to tumour		
phenotype of mouse	+	−	total
fused	5	33	38
normal	35	3	38
totals	40	36	76

TABLE 7. SURVIVAL TIMES OF BACKCROSS TO P STOCK

	survival time (weeks)							
	2	3	4	5	6	12	17	total
normal mice	2	18	9	5	1	0	0	35
fused mice	0	1	1	0	1	1	1	5
total	2	19	10	5	2	1	1	40

(2) *With* dba *tumour* P 1534

The neoplasm used (p 1534) will stimulate antibodies in C 57 black mice. Unfortunately, the sera obtained were not of adequate strength for use with hybrids, so that the results are for tumour inoculation alone. Two backcrosses were tested; to the P strain (76 mice) and to the C 57 blacks (20 mice). The results of the former are shown in table 6. It is clear that the linkage is close. Reference to table 7 makes it seem very unlikely that three of five 'susceptible' fused are cross-overs, since they died so late. The other two may well have been. This is not certain; it may be that the antigen in *dba* tumour P 1534 is weaker than that in the A tumours and the animals failed to respond. The indicated cross-over rate is somewhere between 0 and 5 %

Tables 5 and 6 indicate a low proportion of normal overlaps from the cross $(dba \times CA) \times P$. The ratio of fused to non-fused is 38 : 38. Also, and more significant, there are only three normal resistant mice (which must be either cross-overs or normal overlaps) out of the total of seventy-six, as compared with twenty-four out of 177 for the other crosses (tables 4 and 6). The difference is probably significant

($P = 0.02$). This result is more unexpected because thirty-three of the seventy-six came from crosses in which the female was the heterozygous parent. Of the other crosses lumped in table 5, the cross $\male (A \times CA) \times \female C 57$ gave the highest proportion of normal resistant mice, namely, twelve out of fifty.

The cross $(dba \times CA) \times C 57$ gave twenty mice, eight non-fused and twelve fused, but all resistant. The segregation of one or more genes for resistance in addition to h_2 is indicated in this cross.

<center>DISCUSSION</center>

It is a striking fact that in both the rabbit and mouse, antigens are linked to genes influencing skeletal growth, the linkage in the mouse being very close. In the latter case fused is also closely linked to other genes affecting the same system, namely, the remarkable T, t', t^0 series so thoroughly investigated by Dunn and his co-workers (see Dunn & Caspari 1945; and Gruneberg 1943, pp. 191-199).

There are three difficulties in the detection of cross-overs in the present experiments. The first concerns the incomplete penetrance of fused to which allusion has already been made. Under normal circumstances this would have been solved by genetic tests. The other two are inherent in any work concerned with tumour transplantation. A tumour may kill an animal in spite of antigenic differences capable of eliciting a defensive reaction, tumour 15091a may do so in mice from a number of strains in the presence of very high titres of antibodies (Gorer 1947). It is rather surprising that the ratios obtained with this tumour were so good.

One may also obtain errors of a precisely opposite type. This was first shown by MacDowell & Richter (1932) with their leukaemia, Line I. This gave excellent agreement with the expectation for a single-gene ratio; however, further backcrosses showed that some animals genetically hh had in fact succumbed, the probable explanation of which has just been given. On the other hand, some that were proved to be Hh had survived. Reference to table 3 of the present paper shows that three animals classified as $H_2 h_2$ also did so. One of these gave rather weak reactions and may have been wrongly classified; the other two gave extremely clear-cut results.

Serological work gives the probable explanation. It has been pointed out that both the A strain tumours (particularly $C 1300$) may elicit antibodies other than anti-II. This might be attributed to non-specific stimulation but for the fact that these bodies are readily absorbed by the tumours. It appears, therefore, that a tumour may give a single-gene ratio but contain more than one antigen. It may be that the other antigens are much reduced in amount or they may be much less effective by virtue of their chemical nature. Thus if we could perform this type of experiment in man we might get a tumour containing R_0 (or D) and M that gave a single-factor ratio; however, a few people appear to be able to form anti-M and could thus give an anomaly of the type just described.

It now remains to be considered whether the gene concerned with the dba tumour is linked to H_2 or is an allele. Serological tests show that the dba strain contains an antigen closely related to II. Table 8 shows a serum that gives strong agglutination of both A and dba red cells. It will be noticed that absorption with dba cells removed nearly all the agglutinins for A cells. Other sera have been found containing anti-

TABLE 8. THE ANTIGENIC SIMILARITY BETWEEN *A* AND *dba* RED CELLS

tested on cells of	serum anti-15091*a* diluted one in							
	2	4	8	16	32	64	128	256
A	+ ±	+ +	a.c.	a.c.	a.c.	+ + ±	+	−
dba-2	−	−	+ +	+ + +	+ +	+	−	−

absorbed with cells of	serum tested on *A* cells after absorption (at 1/8)					
	dilution of serum					
	8	16	32	64	128	256
A	+	−	−	−	−	−
dba-2	+ +	−	−	−	−	−

bodies that will not agglutinate *dba* cells, but which are absorbed thereby. It was shown some time ago (Gorer 1936) that the *CBA* strain also contained an antigen related in a similar manner to II. Recently, this has been shown to be true of the *C3H* strain as well. All these antigens differ from one another so there may be a long series of alleles at this locus. Tentatively we suggest that the *dba* gene studied here should be considered an allele and designated H_2^d.

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The electrical properties of the muscle fibre membrane

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An analysis is made of the electric properties of frog muscle using the 'rectangular pulse' technique of Hodgkin & Rushton (1946). The experiments were made on isolated fibres and small bundles of the M. adductor magnus and on the M. extensor longus dig. IV. Sub-threshold currents of about 0·1 sec. duration were applied and wave form and attenuation of the extrapolar potential changes determined.

In the vicinity of the cathode the relation between current and voltage across the fibre membrane is non-linear, and there is evidence of a local electric response with currents of more than 30 % threshold strength. At the anode, however, the membrane behaves as a conductor of approximately constant resistance (§ A).

At an average temperature of 22° C, the following mean values were obtained:
(a) Fibre diameter: 75μ in the bundles of M. adductor magnus, and 45μ in M. extensor longus dig. IV.
(b) Characteristic length of the muscle fibres. 0 65 and 1·1 mm. respectively.
(c) Membrane time constant. 9 and 18 5 msec.
(d) Specific resistance of the myoplasm. about 230 Ωcm.
(e) Transverse resistance of the fibre membrane: 1500 and 4300 Ωcm².
(f) Membrane capacity: about 5 μF/cm².
The numerical differences between isolated fibres and whole muscle arose chiefly from a different value of the membrane resistance, the significance of which is discussed (§§ B 1 and D).

The value of the membrane capacity of muscle is about five times higher than that reported for various other cell membranes and confirmed here for isolated crustacean nerve fibres. The large membrane capacity must be an important factor in determining the slow electrical time scale of muscle

The relations between the electric constants of the resting muscle fibre and some of its physiological properties (time factor of excitation, propagation velocity, rate of decline of the end-plate potential) are discussed (§ E).

The location of the membrane, or interface, at which electromotive changes occur is discussed, and a number of reasons are given which indicate that the site of the electrotonic potential changes must be at the surface of the muscle fibres.

Introduction

In a recent paper Hodgkin & Rushton (1946) have described a method by which the electric constants of a non-medullated nerve fibre could be determined. This method has been applied to frog muscle with the object of measuring the resistance and capacity of the fibre membranes. An absolute measurement of these quantities is of interest for two reasons. First, frog muscles are used extensively to study the rates at which ions exchange between cell and surrounding fluid (Boyle & Conway 1941; Steinbach 1944; Krogh 1946), and one may expect to find a quantitative relation between the permeability to ions and the electric conductance of the cell membrane (see Cole 1940). Secondly, the resistance and capacity of the membrane are of great physiological importance in all excitable tissues in that they influence the rates at which potential changes develop and spread along the fibre surface. For instance, the time factor of electric excitation, the velocity of the action poten-

tial wave and the time course and spatial spread of the end-plate potential must all depend upon the magnitude of membrane resistance and capacity.

Muscle fibres are known to be relatively slow in their electric reactions: the impulse velocity is 10 to 20 times less than in the motor nerve (Adrian & Owen 1921; Schaefer 1936), and the time factor of excitation is about 20 times larger (Lucas 1907/8; Rushton 1930; Schaefer, Schölmerich & Haass 1938; Blair 1941). Even if we compare muscle with thinly medullated nerve, we find that the propagation velocity in a frog muscle fibre of 75μ diameter is less than one-half that in a 30μ crustacean axon. It will be shown that the slower electrical time scale of skeletal muscle fibres can be attributed to their larger membrane capacity.

The experiments were made on isolated fibres or fibre bundles as well as on whole muscles. It will be necessary to describe in detail the results obtained with both types of preparations, for each had its particular advantages and limitations.

METHOD

(1) *List of symbols*

r_i = internal resistance of the fibres per unit length (Ω/cm).

r_o = resistance of the outside fluid per unit length (Ω/cm.).

r_m = transverse resistance × unit length of the fibre membrane (Ω cm.).

R_i = specific resistance of fibre interior (myoplasm) (Ω cm.).

R_o = specific resistance of outside fluid (Ringer solution) (Ω cm.).

R_m = transverse resistivity of membrane (Ω cm.^{2}).

C_m = membrane capacity (μF/cm.^{2}).

λ = characteristic length of fibre = $\sqrt{[r_m/(r_i+r_o)]}$ (cm.).

τ_m = $R_m C_m$ = time constant of membrane (μsec.)

m = $r_i r_o/(r_i+r_o)$ = parallel resistance of inside and outside resistance per unit length (Ω/cm.).

V_A = electrotonic potential at the polarizing electrode (V).

I = current flowing through the polarizing electrode (A).

y = V_A/I = 'electrotonic resistance' (Ω).

x = extrapolar distance (cm.).

Vol.$_t$ = total volume of muscle (c.c.).

Vol.$_i$ = internal volume of fibres (c.c.).

Vol.$_{ext.}$ = outside fluid volume (c.c.).

ρ = radius of fibres (cm.).

l = length of fibres (cm.).

N = number of muscle fibres in parallel.

S = membrane area per unit length (cm.).

(2) *Apparatus*

The method of measuring the electric characteristics of muscle fibres is identical with that described by Hodgkin & Rushton (1946) for lobster nerve. Unless special mention is made, their procedure and nomenclature was adopted in the present work. The technique consists essentially in the application of a subthreshold 'rect-

angular' current to the fibres, and in an analysis of the potential changes produced in the extrapolar region. The circuit shown in figure 1 (*a*) differs from that of Hodgkin & Rushton (1946) only in the use of a mechanical contact breaker and of a balanced earth point in the polarizing circuit. In this figure the electrodes and earth point have been arranged so as to record extrapolar potential changes. To complete the measurements, the polarizing current was monitored across R_6, then leads E_3 and E_4 were reversed to determine the value of m. Each procedure required, of course, resetting of the earth point G.
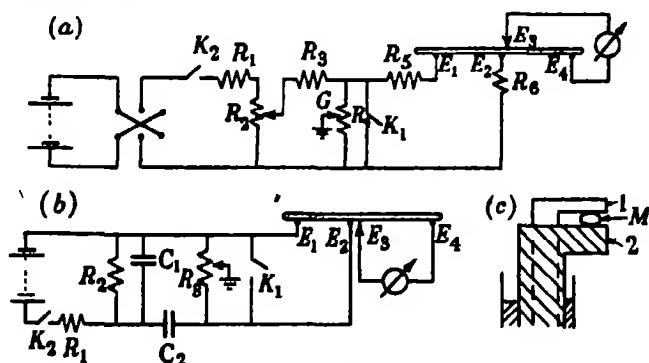
Figure 1. (*a*) Measurement of extrapolar potentials. Electrodes as arranged for isolated fibres. K_1, K_2, keys of Lucas rotating contact breaker; R_1, 15,000 Ω; R_3 and R_5, 5000 Ω; potentiometers; R_2, 60,000 Ω; R_5, 0·5 to 5 megohms; R_6, monitor resistance of 10,000 to 100,000 Ω; E_3, sliding electrode; E_2, E_4, input leads to cathode follower grids. (*b*) Brief double condenser pulses. Electrodes as arranged for M. extensor, dig. IV. K_1, manual short-circuit; K_2, key of contact breaker; R_1, 20,000 Ω; R_2, 2000 Ω; R_5, 5000 Ω; C_1, 0·1 μF, C_2, 0·05 μF. Monitor resistance not shown. (*c*) Electrode connector of E_3 used for M. extensor dig. IV. 1, bridge of filter paper; M, muscle; 2, sharpened wooden connector.

In some experiments, a brief subthreshold current pulse was applied to the muscle, using a double condenser discharge as shown in figure 1 (*b*). The time course of the discharge was adjusted so that it should imitate the brief action of the nerve impulse in a curarized preparation (Eccles, Katz & Kuffler 1941).

The muscle was connected via two cathode followers to a D.C. amplifier of the type described by Hodgkin & Huxley (1945). The two channels of the amplifier were balanced to within 1 : 200, and this, together with the use of a balanced polarizing current, eliminated most unwanted artefacts. In some experiments the contact breaker was coupled by a small condenser to the output of the cathode follower in order to mark the beginning and end of the rectangular current with a brief spike. The grid current in the input circuit was less than 10^{-9} A, and usually about 10^{-10} A. The recording speed in these experiments was relatively low, for the time constant τ_m of the membrane was of the order of 10 to 20 msec. For this reason the delay introduced by stray capacities of the apparatus was negligible. When single fibres were used, resistances of a few megohms were encountered in both polarizing and recording circuits, but even under these adverse conditions the lag of the whole apparatus (including all connexions from contact breaker to cathode ray tube) could be represented by an exponential curve with a time constant of less than 70 μsec. and could thus be disregarded. The time base was calibrated with 50 cycle A.C.

(3) *Preparation and procedure*

Small bundles containing 1 to 4 muscle fibres were isolated from the M. adductor magnus, or M. adductor longus, of English *Rana temporaria*. The muscle was left attached to the bones, or tendons, at either end. The dissection was carried out in a Ringer bath to which usually some curarine was added. This does not affect the excitable properties of the muscle membrane (Lucas 1907/8; Schaefer *et al.* 1938), but it reduces the risk of injuring the muscle fibres at an advanced stage of the dissection when the last nerve branches are severed. In some experiments, the solution was replaced by ordinary Ringer before the fibres were mounted on electrodes.

During dissection, the muscle was placed on a glass slide as described by Ramsay & Street (1941), and its attachments were tied to rubber bands. When a clean stretch of 10 to 15 mm. had been obtained, the preparation was gripped at both ends by screw-controlled forceps and raised into a layer of light liquid paraffin. Electrodes similar to those described by Hodgkin & Rushton (1946) were then applied and their distances measured with an eyepiece scale. The sliding lead E_3 was placed opposite the polarizing electrode E_2, and the polarizing circuit was balanced by temporarily connecting E_4 to earth and adjusting G for zero deflexion. E_4 was then reconnected to its input grid and a preliminary series with varying current intensities of alternating directions was made. After threshold had been determined, a suitable current strength, usually near threshold, but with anode at E_2, was selected and the required measurements were made. At the end of the experiment, the preparation was transferred to a Ringer bath, and the fibre diameters were measured with a 48-fold magnification.

A few comparative experiments were made on isolated nerve fibres of *Carcinus maenas*, using the same apparatus but replacing Ringer by sea-water electrodes.

It was found on several occasions that isolated muscle fibres survived well in a Ringer bath and gave vigorous responses after 24 hr. soaking, yet after being transferred to paraffin oil and mounted on electrodes, would fail to conduct within 1 or 2 hr. It is not clear whether this was the result of the immersion in paraffin oil or of the handling during the experiment, but it raised doubts as to the physiological condition of the fibre membranes. Comparative experiments were therefore made on whole muscles which should give one greater assurance concerning their physiological state. After preliminary work on the sartorius, the M. extensor longus dig. IV was used. This muscle is of cylindrical shape, about 15 mm. long, often less than 0·5 mm. thick, and consists of approximately 50 parallel fibres (variations between 15 and 70 fibres were observed, the former case apparently being an anomaly). In transverse sections of the muscle two or three spindles were frequently seen, and it is possible that these structures introduced some complication. But as they occupy only a few per cent of the total cross-sectional area, they are not likely to affect the extrapolar potential seriously. At times, two or three fibres of the muscle were found to diverge and join another tendon, and it was then difficult to avoid injuring them. These fibres were either carefully removed from the rest of the muscle, or the preparation was discarded. The muscle was mounted in a moist paraffin-wax

chamber. The electrodes were calomel half-cells connected to the muscle by pools of Ringer and sharpened strips of wood which had been soaked in Ringer's solution. One of the wooden connectors was clamped to an ebonite rod which could be driven, parallel to the muscle, by a coarse micrometer. The dimensions of the muscle were measured with a binocular microscope and eyepiece scale.

(4) *Sources of error*

The analysis of the results is based on the theory of a uniform cable of linear dimensions with distributed capacity and leakage resistance (Hodgkin & Rushton 1946; Lorente de Nó 1947). In practice the conditions of the experiment conformed only approximately to the theoretical assumptions, and it is necessary to consider the errors which may have arisen in this way.

Thickness of muscle. The theory demands that the current flow should be strictly parallel in the internal and external conductors, and that there should be no transverse potential differences (p.d.'s) except between inner and outer surface of the membrane. This can be attained only if the length constant λ is very large compared with the thickness d of the tissue (Hodgkin & Rushton 1946). In an average experiment, λ/d was about 4 to 5. To check whether this was a tolerable ratio, two empirical tests were employed.

(*a*) If there were an appreciable transverse potential gradient in the external or internal medium, it would cause a part of the extrapolar potential to be established instantly. This would form a discrete component of the recorded potential change, added to the electrotonic membrane potential which develops gradually and is progressively slowed as lead E_3 is moved away from the polarizing electrode. Furthermore, the first component should reverse sign when E_3 is placed on the opposite side of the muscle, while the membrane component would not reverse. Finally, the membrane potential should be abolished by chloroform, while potential gradients in the fluid conductors would remain. In isolated fibres and the extensor dig. IV, only the 'membrane' component was observed, which indicates that these preparations were sufficiently thin. With the sartorius muscle, however, both components showed up markedly (§ C).

(*b*) The effective thickness of the tissue can be reduced to one half by applying the polarizing current to both sides of the muscle instead of only one. This was done by means of a bridge of filter paper which touched the upper surface of the muscle as well as the wooden electrode (figure 1 (*c*)). If the spread of the extrapolar potential is altered significantly by this procedure, then the ratio λ/d is evidently not large enough. The tests made with this method will be described in more detail below: they confirmed that the M. extensor longus dig. IV was a satisfactory preparation for the present purpose, while the sartorius was not. In the experiments described in § D, the double-sided contact of electrode E_3 was used throughout.

Width of and distance between electrodes. In the ideal case, electrodes E_2 and E_3 should have infinitesimal width (δ), and their distance D from electrodes E_1 and E_4 should be infinite. In practice, the ratio λ/δ was about 5 to 10, and the ratio D/λ not less than 4 to 5. These imperfections are not likely to vitiate the measurements

of length and time constants (λ and τ_m) by more than a few per cent, and no attempt was made to correct for them.

When the extensor dig. IV was used, the arrangement of electrodes E_2 and E_3 (figure 1 (*b*)) inevitably compromised the measurement of the potential change at the polarizing electrode itself: for when E_2 and E_3 made contact, polarizing current flowed through the common part of the electrode and produced a small p.d. in the recording circuit. Furthermore, the electrotonic potential recorded at this point suffered an appreciable decrement, for the combined width of E_2 and E_3, and of the droplet of fluid forming at the point of contact, amounted to about 0·4 mm. This affects the measurement of the constants y and r_i/r_0 (cf. Hodgkin & Rushton 1946), but an approximate correction can be made by extrapolating the observed relation between $\log V_1$ and x to a distance of 0·4 mm. × $\sqrt{[r_i/(r_i+r_0)]}$, which was approximately 0·3 mm.

Distribution of fibres. The theoretical treatment deals with a single uniform cable, but it can equally be applied to a bundle of identical cables arranged in parallel with conducting interspaces. In most experiments we have an aggregate of fibres of very different diameters. In the extensor dig. IV, for example, variations in fibre size of 3·5:1 (25 to 90μ) are common, and extreme variations of 10:1 (10 to 100μ) have been observed. The electric constants derived from such a non-uniform assembly must be treated with caution. They may be regarded as the properties of an 'average' fibre, but not much confidence could be placed on this statement, if it was not supported by measurements on isolated fibres.

Measurement of fibre size. The most serious technical error arose from inaccuracies in determining the fibre diameters. These inaccuracies were due to variations of diameters along the muscle, to elliptical rather than circular cross-sections of fibres and to uncertainty regarding the thickness of adventitious tissue. In extreme cases the error may have been as high as ± 20 %, and one may regard ± 10 % as an average estimate. Fortunately, some of the relevant constants, e.g. λ and τ_m, are independent of this measurement, but others, such as the absolute values of R_m and C_m, are affected by it. The value of R_i is proportional to the square of the diameter, and is therefore the least reliable quantity which has been measured.

In the case of the M. extensor dig. IV the method used for determining surface area and internal volume of the fibres needs further description. The dimensions of the muscle were measured with a low-power microscope, and the fibres were counted after fixing and sectioning. The two required quantities are (i) the volume per unit length, Vol $_i$/1, and (ii) the membrane surface S per unit length, of all the muscle fibres. From the electrical measurements the longitudinal resistance of the outside fluid was known. Using this result and the specific resistance of Ringer solution, the outside fluid volume was calculated and was subtracted from the optically determined total volume to find the internal fibre space. This procedure takes no account of poorly conducting material on the outside of the fibres such as connective tissue fibrils, hence the estimate of the internal volume is somewhat too large.

The total membrane surface could be calculated easily if all fibres had the same diameter: it would then simply follow from $S = 2\sqrt{(\pi N \text{ Vol.}_i/1)}$, where S is total membrane area per unit length, Vol.$_i$/1 total internal volume per unit length, and N the number of fibres. Because of the large scatter of fibre sizes, this formula must
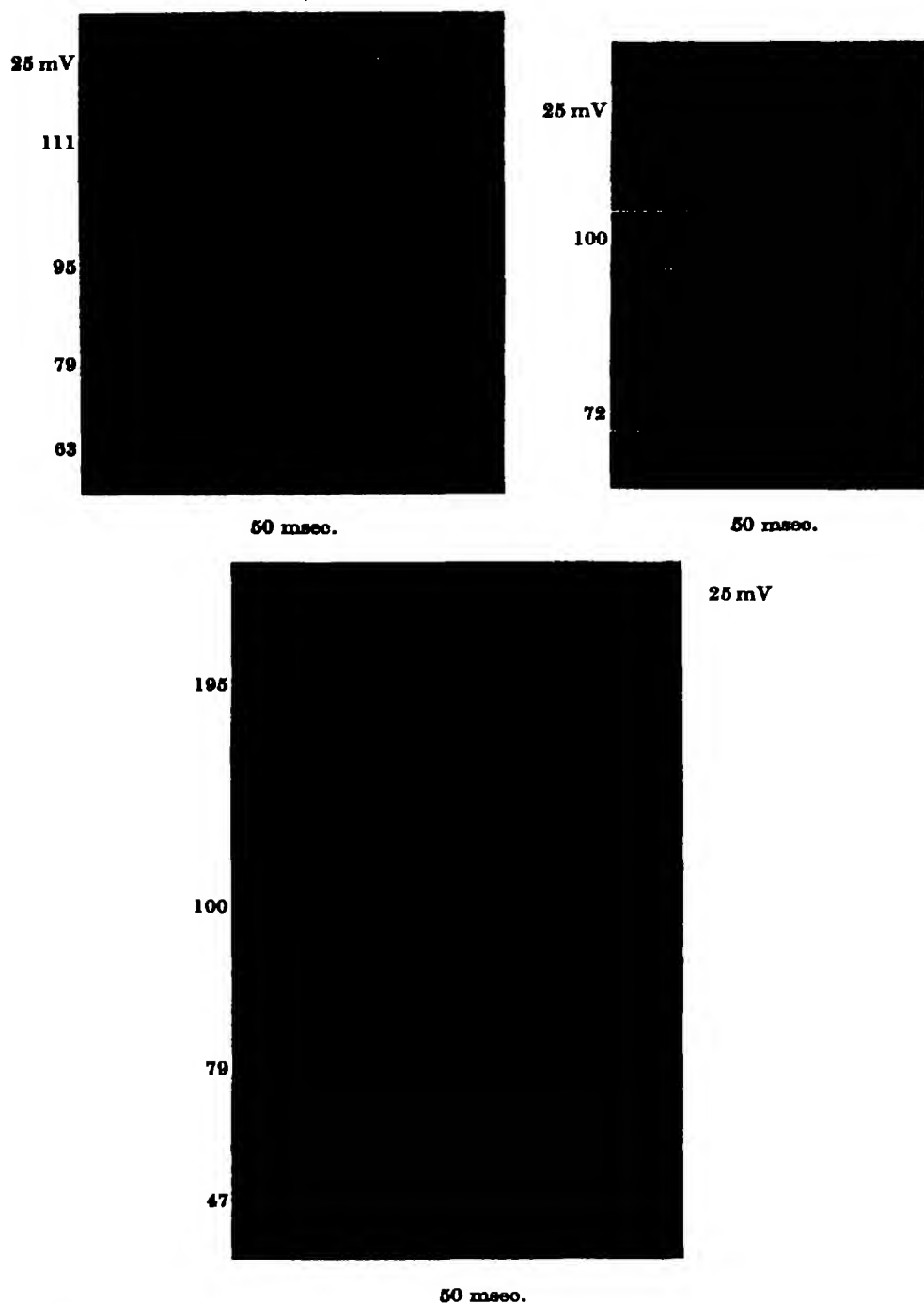
FIGURE 2. Potential changes at the polarizing electrode. Cathodic potentials shown as upward deflexions. Strength of current indicated in relative units. Calibration: 25 mV and 50 msec. A, single muscle fibre. Peaks of diphasic spikes are marked by dots. B, four muscle fibres. Spike potential (not shown) 85 mV. C, anomalous response of a muscle fibre, after a block had developed near the recording electrode.

give too high a value. The order of magnitude of this overestimate was determined in one muscle by measuring the diameters of its 57 fibres. It was found that the true value of S was about 10 % less than calculated from the above equation. As the scatter of fibre diameters appeared to be much the same in all preparations, a reduction factor of 0·9 for the calculated value of S was employed throughout.

In the case of *Carcinus* axons, the outside diameter of the fibre was measured while it was mounted on electrodes in paraffin oil. The internal volume was obtained as for the M. extensor dig. IV, by subtracting the electrically determined outside fluid space. This results in an overestimate of the axon diameter, but no further correction was attempted.

The errors already discussed are of a technical nature: in addition there were a number of small inaccuracies arising from genuine divergences between the behaviour of nerve or muscle and the simple cable theory These will be discussed under the appropriate headings below.

(5) *Control experiments on dead tissue*

To ensure that the electrotonic potentials were correctly attributed to the living cell membranes, the muscles were treated with chloroform. This was applied to isolated fibres by gently stroking their surface with a fine glass rod which had been in contact with a chloroform-saturated Ringer solution. Extensor muscles were exposed to chloroform vapour which was introduced into the moist chamber by means of a pad of filter paper. Within a short time the extrapolar potential changes disappeared, except for a small rectangular wave recorded at the polarizing electrode which did not exceed a few per cent of the original potential (see also Hodgkin & Rushton 1946). The action of chloroform was as complete in whole muscle as in isolated fibres, the only difference being the rapidity of its onset. The effect on isolated fibres was immediate, while that on the extensor muscle was delayed for several minutes.

RESULTS

A. *The relation between current intensity and membrane potential*

Before we can attempt to measure the membrane conductance of muscle, we must find out whether it obeys Ohm's law within at least a limited range of current intensities. That this can only be approximately so, is indicated by the fact that the A.C. impedance of muscle varies continuously with the polarizing current strength (Dubuisson 1935; Katz 1942), and that subliminal currents produce a local response at the cathode (Kuffler 1942). Nevertheless, direct measurements show that the relation between electrotonic potential and applied current is not far from linear with cathodic currents of less than one-third threshold and with anodic currents of considerably greater intensity (figure 4). It is true that a very slight, continuous, upward curvature of the voltage : current relation was sometimes observed (see also Schaefer *et al.* 1938), but as a first approximation the membrane characteristic appears to follow Ohm's law in the range indicated in figure 4.

Near threshold, the relation between catelectrotonic potential and current strength becomes distinctly non-linear. This effect was observed in isolated fibres

as well as in whole muscle (figure 4). There was a significant difference between the time courses of anodic and cathodic potentials, the latter continuing to rise for a longer time. At threshold, propagated spikes arose after a period of gradual inflexion upwards (figures 2A, B and 3A) and all transitions between the plateau and the inflexion of figure 2B could be obtained by fine adjustment of the current strength. The whole family of potential-time curves is so similar to the local responses in *Carcinus* nerve described by Hodgkin & Rushton (1946) and Hodgkin (1947) that no further comment is required.
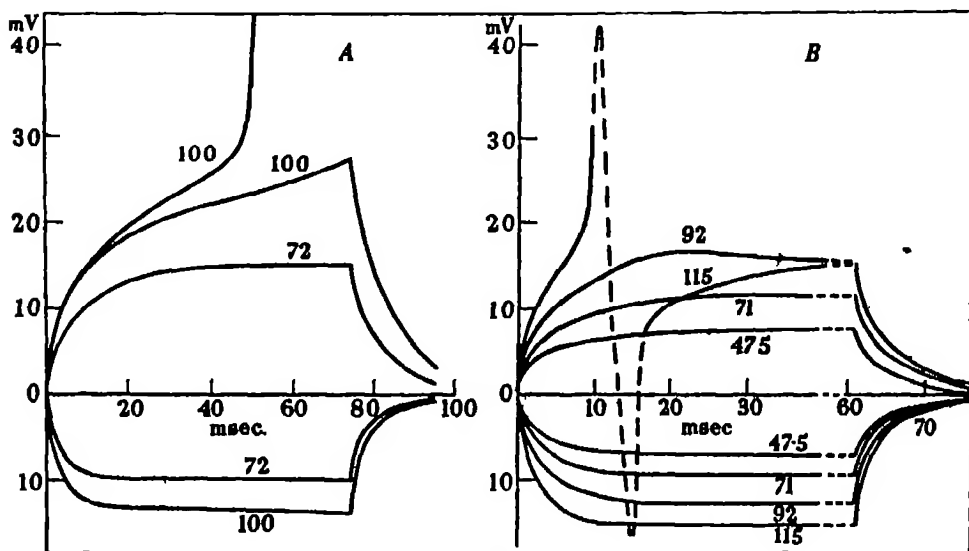
FIGURE 3. Superimposed records of potential changes at the polarizing electrode. Cathodic potentials shown as upward deflexions. Relative current strength shown in figure. A and B, two experiments on bundles of four fibres.

In several preparations, a different type of local response was seen. It consisted of an initial hump, rather than a prolonged 'creep' of the catelectrotonic potential. In one case, illustrated in figure 2C, the hump increased in size and became oscillatory as the current intensity was raised. After the local responses had died out, the electrotonic potential settled to a lower level than at the anode. A behaviour of this kind was observed in fibres of low excitability, and it sometimes developed in the course of an experiment. Although it cannot be regarded as characteristic of normal muscle, a very similar type of reaction has previously been described in the giant axons of cephalopods (Arvanitaki 1939; Cole & Curtis 1941) where it appears to be the usual event.

In small bundles of fibres the inflexion of the local response occurred at about 20 mV (varying in 7 experiments between 16 and 32 mV). The size of the spike varied between 35 and 85 mV. Conduction velocity was determined from the two peaks of the diphasic response and, in 5 experiments at 22° C, was 1·0 to 1·45 m./sec., with a mean of 1·27 m./sec. This is considerably less than the velocity in whole

sartorius muscle (1·6 m./sec. at 20° C, see Eccles *et al.* 1941). There are probably two reasons for this difference: first, one would suspect the isolated fibres to have a reduced 'safety margin' and therefore to conduct at an abnormally low rate (see Method). Secondly, the resistance on the outside of the fibres was very high (see § B 4), and this must cause a considerable drop in velocity (cf. Hodgkin 1939; Katz 1947 b). For example, a 60μ fibre which conducted at a speed of 1 m./sec., had a ratio of outside : inside resistance of 1·02: the velocity of this fibre *in situ* would have been $1·0 \times \sqrt{[(r_i + r_o)/r_i]} = 1·42$ m./sec.

FIGURE 4. 'Voltage/current' relation at the polarizing electrode. *A*, from the experiment of figure 3 *B*. *B*, from a M extensor dig. IV. Ordinates: maximum amplitude of local potential change in mV. Abscissae: current strength in μA Threshold of propagating spikes indicated by arrow. *Note.* In experiment B the deflexions were steady, while in A, with currents near threshold, the amplitude of the cathodic potential began to decline before the break of the current (cf. figure 3 *Ḃ*).

B. *Electric constants of isolated muscle fibres*

It follows from these observations that the muscle membrane gives a local electric response in the region of the cathode which may persist for 100 msec. or more, and this causes the apparent membrane resistance to depart significantly from Ohm's law. To minimize this complication the analysis was restricted to the potential changes at the anode.

Figure 5. Anodic potential changes. *A*, M. adductor magnus, four fibres (diameters 70, 70, 45, 45μ). *B*, M. adductor magnus, two fibres (diameters 130 and 50μ). *C*, *Carcinus* nerve. Single fibre of 43μ outside diameter. Extrapolar distances shown in mm. Instant of current make is indicated by a dash. Time marks at 20 msec. interval.

(1) *Extrapolar decrement and the value of* λ

In figures 5*A* and *B*, the extrapolar potentials are shown in two preparations containing four and two muscle fibres respectively. There are considerable differences in the spatial spread and time course of the anelectrotonic potentials. In figure 5*B*, the potential spreads *farther* and rises and falls more *slowly* than in figure 5*A*. This indicates that in the former case the fibres had a substantially higher membrane resistance, while their membrane capacity may not have been very different from that in figure 5*A*.

For comparison, the potential changes in a nerve fibre of *Carcinus maenas* are illustrated in figure 5*C*. It is clear at once that the extrapolar potential spreads *farther* and rises and falls more *quickly* in the 30*μ* nerve axon than in the larger muscle fibres. This suggests that the membrane resistance of frog muscle is rather less, and its membrane capacity considerably larger, than in crab nerve.
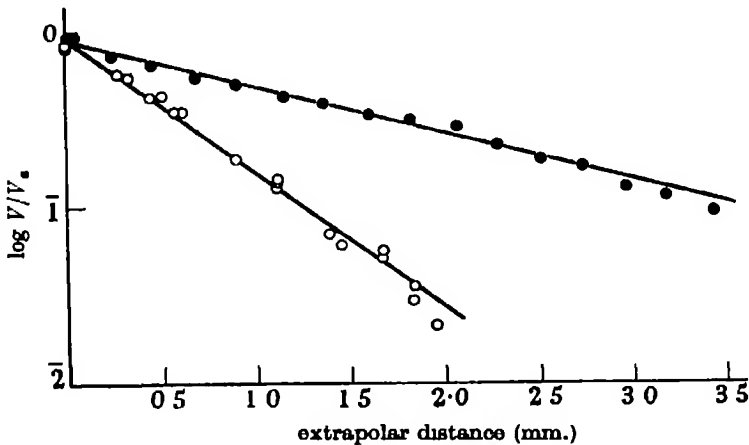
FIGURE 6. Extrapolar decrement of the steady potential change. Ordinates: $\log_{10}$ of voltage. Abscissae: extrapolar distance in mm. ○, four fibres of M. adductor magnus. Four series of observations. ●, *Carcinus* axon. Two series of observations.

To find the magnitude of these membrane constants, the next step is to determine the characteristic length λ and the time factor τ_m of the fibres. In figure 6, the final value of the extrapolar potential is plotted logarithmically against distance. There is no serious divergence from a linear relationship which shows that the decrement is approximately exponential as predicted by theory. The value of λ is 0·58 and 1·15 mm. in the experiments of figures 5*A* and *B*, and 1·64 mm. in the *Carcinus* axon of figure 5*C*.

The results of 9 experiments on small groups of muscle fibres are shown in table 1. The mean value of λ was 0·65 mm. and it varied between 0·47 and 1·15 mm.

The figures of table 1 are unselected results from muscle fibres capable of conducting impulses. The physiological conditions, however, of these fibres varied considerably. Some survived a prolonged experiment well, while others failed to conduct within 1 or 2 hr. after mounting in liquid paraffin (see Method), and the membranes of these fibres may have been abnormally leaky. In calculating mean

TABLE 1. ISOLATED MUSCLE BUNDLES

'weight'	number of fibres	fibre diameter (μ)	temperature (° C)	λ (mm.)	τ_m (msec.)	R_i (Ω cm.)	R_m (Ω cm.²)	C_m (μF/cm.²)	percentage external fluid space
2	3	100, 100, 30	21·5	0·48	4·6	280	1030	4·5	11
1*	2	70, 70	28	0·47	6·5	131	650	10	15·5
4	4	70, 70, 45, 45	23	0·58	5·9	150	1010	6	22
3	4	90, 90, 90, 45	21	0·59	7	182	1370	5	12
1	3	120, 80, 80	21	0·91	13	266	2000	6·5	21
1	2	130, 50	22	1·15	27	175	4500	6	10·5
4	4	95, 75, 45, 30	23	0·66	7	151	1500	5	19
2	1	65	21	0·78	15	152	2100	7·3	17·5
1	1	60	22·5	0·7	6·5	220	1510	4·3	26·5
weighted mean	(1–4)	75	22·5	0·65	9	176	1500	6	17

* This preparation had been subjected to prolonged accidental stimulation.

values, the experiments have been 'weighted' as shown in column 1 of table 1, taking into account (i) consistency of threshold, (ii) clean appearance of fibre surface, and (iii) number and consistency of individual observations.

There were no significant differences between experiments on single fibres and on mixed bundles of 2 to 4 fibres. The values of λ observed in the two single-fibre experiments were close to the mean value of all the other experiments. In view of the marked disparity of fibre sizes in small muscle bundles, one might have expected some divergence from the exponential decrement, but no serious deviation was found (figures 6 and 9). There appear to be two explanations for this result: (a) The contribution which the small fibres make to the extrapolar potential may not be large enough to produce a significant departure from the exponential decay. (b) As Hodgkin & Rushton (1946) have pointed out, the membrane conductance per sq.cm. might be less in a fibre of smaller diameter. Such a relation is indicated by measurements on a variety of non-medullated axons (table 7), and it is easy to see that if muscle cells of all sizes are to come into ionic equilibrium with their environment in approximately the same time, the conductance of the resting cell membrane must vary in proportion to the volume/surface ratio. If a relation of this kind holds, the scatter of λ in fibres of different size would be greatly reduced.

(2) The time constant τ_m

The time constant of the membrane was determined by two methods: (i) by plotting the half-times of rise and fall of the extrapolar potential against distance; and (ii) by analyzing the potential change at the polarizing electrode (figure 7). The first procedure depends upon an empirical relation found by Hodgkin & Rushton (1946), viz. that the half-value of the electrotonic potential propagates with constant velocity which is equal to $2\lambda/\tau_m$. It is a fairly accurate method for it takes account of a series of measurements and is not subject to the errors introduced by the finite width of the electrodes.

Because of these errors the second method is likely to give an overestimate of τ_m. This seems to be borne out by the results, for the mean value of τ_m obtained by this

method was 25 % greater than the mean of the 'half-time' measurements. The figures in table 1 represent the average results obtained by both methods.

In some experiments the anelectrotonic potential showed a slight prolonged creep which is seen, for instance, in figure 3 *A*. A similar effect was seen occasionally in whole muscle (cf. also Schaefer *et al.* 1938). It was clearly due to some secondary change of the membrane potential which is not explained by the simple cable theory, but the effect was small and did not appreciably alter the result of the analysis.
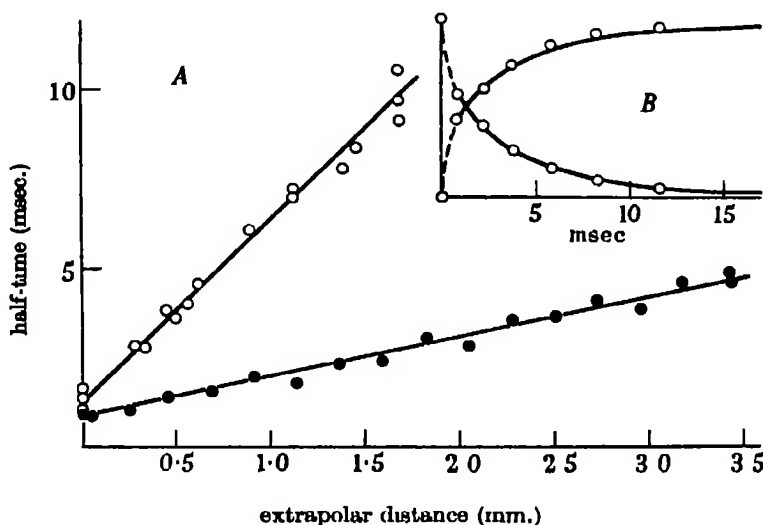
FIGURE 7. Determination of τ_m. *A*, 'propagation velocity' of half-value of the extrapolar potential. Ordinates. time required for rise, or fall, to one half of the steady deflexion. Abscissae extrapolar distance. Same experiments as in figure 6. Each point represents the mean of two determinations made on rise and fall of the record. ○, four fibres of M. adductor magnus Four series of observations. ●, *Carcinus* axon Two series of observations. *B*, potential change at the polarizing electrode. Same muscle as in A. The two curves are tracings of rise and fall. The circles are calculated for $X = 0$ and $\tau_m = 5\cdot8$ msec. Method *A* gives $\tau_m = 5\cdot9$ msec.

The mean value of τ_m in all 9 experiments was 9 msec., but there were variations between 4·6 and 27 msec. It will be noted in table 1 that there was a correlation between the values of λ and τ_m in the individual experiments, and it appears that the variations of both quantities arose from an inconstancy of the membrane resistance (see also § B 4).

(3) *The longitudinal resistance of muscle*

The current encounters two kinds of resistance in the muscle: (*a*) the parallel resistance *r* of the interior of the fibres and the surrounding medium ($r = ml$, where *l* is the length of muscle and $m = r_i r_o/(r_i + r_o)$; and (*b*) a resistance *y* due to the back e.m.f. of the membrane at the polarizing electrodes ($y = V_A/I$, where V_A is the steady electrotonic potential at the electrode and *I* the current passing through it) Both values, *m* and *y*, are required for the further evaluation of the resistance and capacity of the membrane.

To find y, the current I was measured by recording the potential drop across a monitor resistance R_2 (figure 1 (a)). The value of m was determined by reversing electrodes E_3 and E_4, recording the voltage gradient at various points of the equatorial region, and measuring the current as before.

In the measurement of m, some inaccuracy arises from small irregularities along the fibre surface, such as the electrode contacts and fine droplets of saline. Their effect is to give rise to a small electrotonic potential which is added to the expected rectangular wave form (see also Hodgkin & Rushton 1946). Another frequent complication consisted of a small but definite overshoot of the potential change at the make and break of the current This could not be explained by surface irregularities or by the imperfections of electrode width and interelectrode distance, all of which produce a 'creep' and not an 'overshoot', nor could it be traced to an artefact. The effect was not observed if a *Carcinus* axon or a silk thread was used, and it was not due to inconstancy of the applied current which was monitored across R_2. Whatever their causes, the net result of all these complications was to introduce some uncertainty, for it was difficult to decide whether the initial or final deflexion of the record should be taken. Fortunately, the error never exceeded 10 %.

If we multiply the value of m by the cross-sectional area of the preparation we obtain the specific resistance of muscle tissue. This quantity may be compared with values previously determined by different methods. Hartree & Hill (1921) measured the longitudinal resistance of chloroform-treated frog's sartorii with alternating current (50 c./sec , 1 mA) and obtained a specific resistance of $159\,\Omega$ cm. at $20°$ C. Bozler & Cole (1935) found that the transverse high-frequency resistivity of a frog's sartorius at $16\cdot5°$ C was about $185\,\Omega$ cm. In table 2, the results of 9 experiments on small bundles of fibres, and of 14 experiments on extensor muscles are shown, the mean values, at $22°$ C, being 156 and $157\,\Omega$ cm. respectively. There is a large scatter, especially in the isolated fibres, but this is almost certainly due to the large error in finding the cross-sectional area of the preparation (see Method).

TABLE 2. SPECIFIC RESISTANCE OF MUSCLE

A. Isolated fibres and small bundles

temperature (° C)	21·5	28	23	21	21	
specific resistance (Ω cm)	228	116	128	160	184	
						mean
temperature (° C)	22	23	21 '	22·5		22·5
specific resistance (Ω cm.)	164	135	135	155		156

B. Extensor dig. IV

temperature (° C)	20	20	23	22	23·5	25	26·5	
specific resistance (Ω cm.)	189	151	182	153	158	151	143	
								mean
temperature (° C)	25	25	22·5	17	18·5	20		22
specific resistance (Ω cm.)	140	129	163	163	169	157		157

As Hartree & Hill (1921) pointed out, the resistivity of frog muscle is approximately equal to that of a 0·36 % NaCl solution. It is clear that with all three methods, only the parallel resistances of external and fibre medium were involved, for the membranes offer no appreciable impedance to high frequency A.C. (Bozler & Cole

1935), nor to any current after treatment with chloroform (see Method, cf. also Guttman 1939), nor are they involved to any appreciable extent with measurements made in the equatorial region (cf. Hodgkin & Rushton 1946). Thus, the high specific resistance of the whole tissue shows that the conductivity of the myoplasm is low, probably rather less than one-half that of the Ringer solution outside.

(4) *The electric constants of muscle fibres*

Having determined the values of λ, τ_m, y and m, and knowing the volume and surface of the fibres, we are now in a position to calculate the relevant constants. For this the following equations are used (see Hodgkin & Rushton 1946).

$$(a) \qquad r_i/r_o = m\lambda/2y,$$

$$(b) \qquad \text{Vol.}_{\text{ext.}}/\text{cm.} = R_o/m(1+r_o/r_i),$$

$$(c) \qquad R_i = \Sigma(\pi\rho^2)\,m(1+r_i/r_o),$$

$$(d) \qquad R_m = Sm\lambda^2(2+r_i/r_o+r_o/r_i),$$

$$(e) \qquad C_m = \tau_m/R_m.$$

(a) The value of r_i/r_o was not very accurate as it was affected by random errors of three measurements. It is, however, a useful quantity for it gives an indication of the amount of external shunting and enables one to calculate the magnitude of the membrane action potential, which is equal to the observed spike multiplied by $(1+r_i/r_o)$. The recorded action potentials in single fibres and in synchronous volleys of small bundles varied between 35 and 85 mV. The calculated action potentials across the fibre membrane were 70 to 120 mV. The upper value is close to the figures given by Hodgkin & Huxley (1945), Hodgkin & Rushton (1946) and Hodgkin (1947) for various non-medullated nerve fibres.

Some of the small fibre bundles had been carefully freed from loose connective tissue, and this seemed to be reflected in a low value of r_i/r_o. In the 9 experiments on isolated bundles, r_i/r_o varied between 0·25 and 0·96, with a mean of 0·47. In 13 experiments on the M. extensor dig IV, r_i/r_o was on the average 1·08 and varied between 0·47 and 1·75. An early experiment on the extensor muscle gave the exceptional value of 3·2, in this case no attempt had been made to drain off excess fluid.

(b) The outside fluid volume is shown in tables 1 and 5. In isolated fibres the external fluid space amounted to 17 % (10·5 to 26·5 %), in the foot muscles to 27 % (17 to 45 %) of the total volume These figures are higher than the 13 % which have been obtained by chemical methods on the frog's sartorius (Boyle, Conway, Kane & O'Reilly 1941). The difference is probably due to the fact that the sartorii had been blotted with filter paper, a procedure which could not be adopted on the present preparations.

(c) The specific resistance R_i of the myoplasm is obtained from the previous data and the cross-sectional area of the fibres. The individual values are probably accurate only within 40 %. The results are shown in tables 1 and 5. In isolated fibres the unweighted mean value at 22·5° C was 188 Ω cm., in the foot muscles it was 255 Ω cm. at 22° C. The average of all 23 experiments at 22° C was approximately 230 Ω cm., i.e. about 2·9 times the resistivity of Ringer solution. By a different method, Bozler

& Cole (1935) arrived at 260Ω cm. at 16·5° C and concluded that the conductivity of muscle fibres is one-third of that of Ringer solution. According to Boyle & Conway (1941), 23 % of the fibre volume is taken up by non-solvent space, hence the specific resistance of the 'fibre water' would be about 180Ω cm. at 22° C.

(d) The value of the membrane resistance depends upon several measurements, though mainly those of λ, m and S. There is little doubt that the observed variations of R_m are too large to be explained by random errors, and they probably reflect the variable physiological state of the fibre membrane (see also Hodgkin & Rushton 1946, Lorente de Nó 1947)

The average value of R_m in small bundles was 1500Ω cm.², and the extreme range 650 to 4500Ω cm.².

(e) The mean value of C_m in these experiments was 6 μF/cm.², varying between 4·3 and 10 μF/cm.². It will be seen below that the membrane capacity of the whole muscle did not differ substantially from this value, but that its membrane resistance was considerably higher.

(5) *Experiments on single nerve fibres of* Carcinus maenas

The specific membrane capacity of muscle fibres is much higher than the value of 1 to 1·5 μF/cm.² which has been found for many other cell membranes (Cole 1940, Hodgkin & Rushton 1946). This suggests that the muscle membrane has a higher dielectric constant or is thinner than the other membranes which have been investigated. Before accepting this conclusion, it was considered advisable to make control experiments on nerve axons of *C. maenas* (see Method).

An example of the results has been shown in figures 5 to 7 which illustrate the fact that the length constant λ is greater and the time constant τ_m smaller in *Carcinus* nerve than in frog's muscle A summary of 3 experiments is shown in table 3 which agrees substantially with the values obtained by Hodgkin (1947). The value of R_i is rather lower than Hodgkin's figure, but this difference cannot be pressed in view of the multiple errors which affect the measurement of this constant. The membrane capacity was 0·72 to 1·75 μF/cm.², with a mean of 1·17 μF/cm². In Hodgkin's 8 experiments, C_m was 0·7 to 2·03 μF/cm.² with a mean of 1·11 μF/cm.². Thus the membrane capacity of *Carcinus* axons obtained in the present experiments agrees satisfactorily with that of other observers, and this lends more weight to the statement that there is a genuine difference in the case of muscle.

TABLE 3. *CARCINUS* NERVE FIBRES

temperature (° C)	outside diameter of preparation (μ)	λ (mm.)	τ_m (msec.)	R_i (Ω cm.)	R_m (Ω cm.²)	C_m (μF/cm.²)
17	42	1 6	2·1	62	2940	0·72
16·5	32	1·81	5·5	36	3150	1·75
16	43	1·64	3·9	66	3700	1·05

C. *Experiments on the whole sartorius muscle*

As the physiological state of the isolated fibres was probably not normal, the experiments were repeated on whole muscle. The limitations which are imposed

by the thickness of the tissue and by the presence of a non-uniform population of fibres have been discussed above (Method). Preliminary experiments were made on sartorius muscles, with the following results.

Effect of thickness of muscle. The extrapolar potential of the sartorius shows two distinct components, a rectangular wave whose sign reverses when the recording electrode is placed on the opposite side of the muscle, and a proper electrotonic potential. The nature of the two components has already been discussed (see also Biedermann 1896-8). The rectangular wave-form arises from the transverse resistance of the muscle volume and, as pointed out by Schaefer *et al.* (1938), it can be balanced by feeding a suitable portion of the applied voltage into the recording circuit. Unfortunately this procedure does not alter the fact that the current flow in this relatively thick sheet of muscle is not parallel, and that therefore a quantitative analysis of the results becomes impracticable. The layer of muscle fibres which makes intimate contact with the polarizing electrode is subjected to a steeper longitudinal potential gradient than the more remote layers, hence the apparent value of λ varies with the position of the fibres which have been selected for measurement This was confirmed by exploring the surface of the muscle with a capillary electrode There was a very sharp electrotonic decrement near the polarizing electrode, but a much more diffuse spread of the potential on the opposite side of the muscle.

If the polarizing electrode is applied to both sides of the muscle, these complications are reduced. In this way the thickness of the tissue is effectively halved, and the situation conforms more nearly to the theoretical case. In table 4, the results of an experiment are summarized. As expected, the apparent value of λ became greater when the polarizing electrode was applied to both sides of the muscle, but there is no certainty that the true value of λ had been reached. The quantities of R_m and C_m in table 4 were computed on the assumption of uniform fibre size (450 fibres of 73μ diameter). The results of series B agreed fairly well with those of tables 1 and 5, but not much confidence could be placed in them, and these experiments were discontinued.

TABLE 4. APPARENT CONSTANTS OF SARTORIUS MUSCLE

	λ (mm.)	τ_m (msec.)	R_i (Ω cm.)	R_m (Ω cm.^{2})	C_m (μF/cm.^{2})
A, E_2 applied to one side	0·55	10·3	218	1180	8 7
B, E_2 applied to both sides	0·8	10·8	248	2080	5·2

Effect of external perimysium. The outer surface of the sartorius muscle is covered by a strong sheath of connective tissue, several microns thick, while the inner surface of the muscle is freely exposed. It was of interest, therefore, to measure the extrapolar potential when electrodes E_{1-4} were applied to alternate sides of the muscle. If the external perimysium were responsible for an appreciable part of the electrotonic potential, then this potential should become much greater if polarizing and recording electrodes are placed in contact with the perimysium, and most of the current is forced to penetrate it, than if the electrodes are on the opposite side of the muscle.

No significant difference, however, was observed, and it appears, therefore, that the external perimysium provides no serious hindrance to the movement of sodium and chloride ions.

D. *Experiments on the M. extensor longus dig. IV*

The same test as used in the experiment of table 4 showed that the M. extensor longus dig. IV was satisfactory for the present experiments (see Method). In an experiment in which the polarizing anode was applied (i) to one side, (ii) to two opposite sides, and (iii) to the four sides of the muscle, no significant difference in the spread of the extrapolar potential was observed, the values of λ being 1·56, 1·61 and 1·44 mm. and the values of τ_m 29·6, 32·2 and 31·5 msec. respectively. Furthermore, the extrapolar potential had no 'rectangular' component except when there was a direct fluid contact between electrodes E_2 and E_3 (figure 1 (b)). To minimize any residual errors which might arise from divergent current flow, the anode was always applied to both sides of the muscle.

mm
0·0

0·3

0·7

1·0

1·5

2·0

50 c./sec.
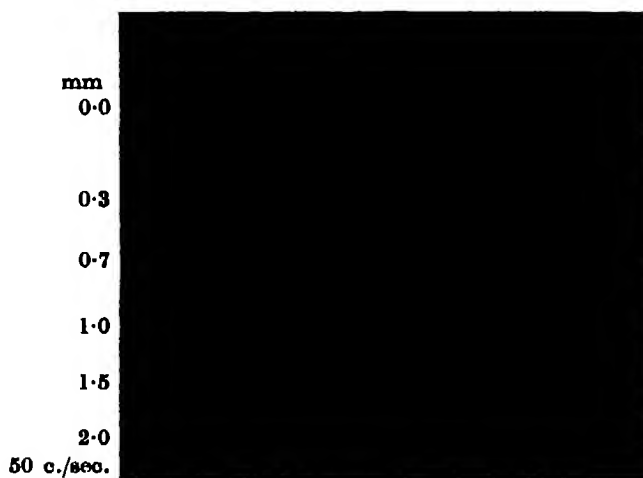
FIGURE 8. Anodic potential changes in M. extensor longus dig. IV. Distance between electrodes E_2 and E_3 shown in figure. Instant of make and break marked by a faint spike. At 'zero distance' a small instantaneous deflexion arose from the common resistance of polarizing and recording electrode (see Method).

The methods of measurement differed from those described above in only one respect, namely, that an extrapolation had to be made when calculating the value of y (see Method). This was necessary because of the appreciable width of the electrode contacts. For the same reason, the value of τ_m was determined by relying solely upon 'half-time' measurements at various distances (§ B (2)), and not upon an analysis of the potential at the contact of E_2 and E_3. These limitations, however, are not likely to diminish the accuracy of R_m and C_m.

An example of the extrapolar potential changes is shown in figure 8. Results of a similar experiment are plotted in figure 9, which indicates that the wave form and

attenuation of the electrotonic potential in these muscles agrees satisfactorily with the cable equations.

The results of 15 experiments are summarized in table 5, to which only a few comments are required.
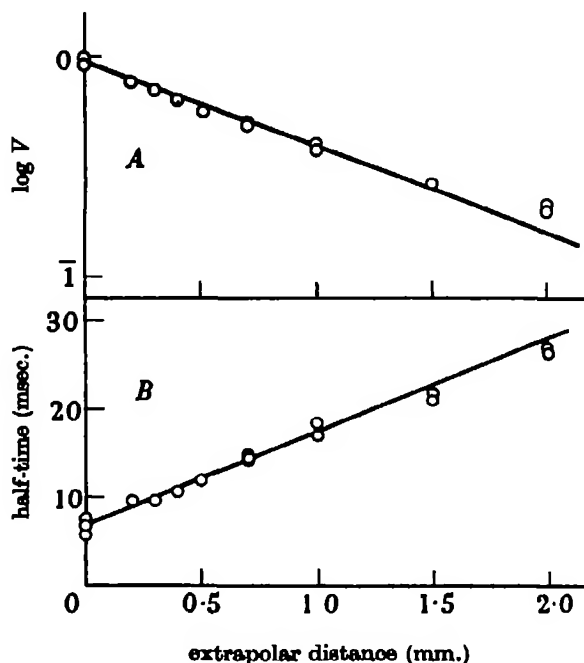
FIGURE 9. Measurement of λ and τ_m in M. extensor dig. IV. *A*, spatial attenuation, plotted as in figure 6. *B*, 'propagation velocity' of half-value, plotted as in figure 7. In each case, two series of observations, made with current pulses of 90 and 220 msec. duration respectively.

TABLE 5. M. EXTENSOR LONGUS DIG. IV

temperature (° C)	λ (mm.)	τ_m (msec.)	R_i (Ω cm.)	R_m (Ω cm.²)	C_m (μF/cm.²)	percentage external fluid space
20	0·97	12	320	2600	4·6	26
20	1·5	30·5	330	9500	3·2	45
22	0 98	15·5	226	3900	4 1	28
23	1·06	11·9	355	4100	2·9	29
23·5	0·88	12·5	234	3000	4·2	21
24	1·45	21·7	—	—	—	—
25	0·75	7·3	222	1500	4 9	25
26·5	1·2	22·5	218	5300	4·3	28
25	0·96	15	218	2900	5·2	27
25	1·34	38	329	6400	5·9	22
25	1·53	20	214	5000	4·0	38
22·5	1·20	25·5	290	6200	4·1	31
17	0·93	11·5	210	3200	3 6	23
18·5	1·04	17	206	4200	4·1	17
20	0·86	13	210	2200	5·8	24
mean 22	1·1	18·5	255	4300	4·4	27

The average diameter of the fibres is considerably smaller than in the thigh muscles, 45μ as compared with 75μ. A similar result was obtained by Mayeda (1890) who gave the following values:

frog's sartorius· mean diameter 84μ, range 19 to 152μ;

extensor longus dig. I: mean diameter 46μ, range 11 to 80μ.

This difference between foot and thigh muscles must be borne in mind when comparing their electric constants, for a correlation between fibre diameter and membrane conductance has been suggested by Hodgkin & Rushton (1946).

The values of λ, τ_m and R_m are, on the average, substantially greater in the extensor muscle than in the isolated bundles of thigh muscles (see table 1). This may reflect a genuine difference between the two types of muscles and would agree with the suggested relation between fibre size and permeability. But it is probable that a major part of the difference in R_m, and in the other constants which depend upon R_m, is to be explained by the better physiological condition of the undivided muscle.

There does not appear to be a marked difference in the values of C_m in tables 1 and 5, and the mean values become more nearly equal if account is taken of the different methods in deriving them. To make the comparison between isolated fibres and whole muscle more precise, the mean value of C_m in table 1 should be replaced by one which is based entirely on 'half-time' measurements. We obtain then for the isolated bundles a mean value of C_m of $5\cdot3\,\mu\mathrm{F/cm}^2$ with a standard error of the mean of $0\cdot55\,\mu\mathrm{F/cm}^2$ (9 experiments), and for the extensor dig. IV a mean of $4\cdot4\,\mu\mathrm{F/cm}^2$, with a standard error of $0\cdot23\,\mu\mathrm{F/cm}^2$ (14 experiments).

E. *The relation of the membrane constants to physiological properties of muscle*

The large capacity of the membrane is probably an important factor in determining the long time-scale of the electric reactions of muscle. This will be considered more fully on three examples. (i) the time factor of excitation of muscle (Lucas's (1907/8) and Rushton's (1930) α-constant), (ii) the conduction rate, and (iii) the end-plate potential in curarized muscle.

(1) *The time factor of electric excitation*

It was first shown by Keith Lucas (1907/8) that the strength-duration curve of a sartorius muscle has two or three components of which only the slowest is due to the muscle fibres. This conclusion was temporarily disputed, but it is now clearly established that the 'chronaxie' of muscle at 20° C is of the order of 10 msec., provided the electrodes have been arranged so as to ensure an approximately parallel current flow and that the points of current entry and exit are at least a few mm. apart (Rushton 1930; Schaefer *et al.* 1938; Ramsay & Street 1941; Blair 1941). Schaefer *et al.* (1938) have recorded electrotonic potentials from a sartorius muscle and concluded that the half-time in the vicinity of the polarizing electrode was approximately equal to the chronaxie. These authors assumed that the development of the electrotonic potential as well as that of the excitation process were governed by a single exponential time factor. This view can only be regarded as a rough approximation, but the general conclusions reached by Schaefer *et al.* (1938) and the order of magnitude of their quantities are substantially correct.

The threshold of electric stimulation is not governed solely by the 'passive' attainment of a certain catelectrotonic potential (see Rushton 1937; Katz 1939; Hodgkin & Rushton 1946). It is evident from figure 3 A that the applied current must be maintained during the period of inflexion until the local regenerative process at the cathode has reached sufficient intensity to propagate itself. Hence, the time factor which is derived from a strength-duration curve does not characterize a simple electrotonic charging process, but a sequence of events of which only the first stage is determined by the resistance and capacity of the resting membrane. The later stages depend upon the character of the local response and involve active changes of the membrane constants. Nevertheless, a large value of R_m and C_m of the resting, or anodically polarized, membrane entails a slow initial development of the excitatory disturbance and must be reflected in a large time factor of excitation.

(2) *The propagation velocity of muscle*

The conduction velocity in a sartorius muscle, at 20° C, is approximately 1·6 m /sec. (Eccles *et al.* 1941). In isolated fibres of 70 to 80µ diameter the conduction rate was about 20 % less, even after allowing for the high external resistance. This difference, however, may be attributed to a state of lower excitability (§A). In the M. extensor dig. IV the propagation velocity was measured after curarization, and values close to that of the sartorius muscle were found. At 20° C, the foot and front of the muscle spike, i.e. presumably the largest fibres, travel at a speed of 2·2 m /sec. The spike summit gave a value of 1·6 to 1·8 m./sec Occasionally, a slow fibre could be stimulated separately from the rest. The lowest speed which could thus be observed was 1·0 m /sec. at 20° C.

These values are lower than those of crustacean nerve axons of the same or smaller diameter (cf. Hodgkin 1939; Prosser 1946) A comparison of conduction rates in different fibres is difficult, because the conduction of impulses depends upon many variable factors, and usually only one or two of these variables are known. Moreover, an entirely adequate theory of propagation has not yet been developed. The local circuit theories of Blair (1934), Rashevsky (1936), Rushton (1937) and Offner, Weinberg & Young (1940) lead to an equation of the type $v = S'L/\alpha$, where v is conduction velocity, L length constant, α time constant of the membrane and S' a 'safety factor' which depends upon the size of the action potential and the excitability of the membrane. These theories are based on the assumption of a sharp boundary between resting and active region, and according to Offner *et al.* (1940), the values of L and α of the *active* region should be used.

If we consider propagation *in situ*, or in a large volume of tissue or saline, we can transform the above equation into

$$v = \frac{S'}{\sqrt{(2R'_m)}} \times \frac{\sqrt{\text{radius}}}{C_m \sqrt{R_i}},$$

where R'_m is the active membrane resistance (Ω cm.²) and C_m the membrane capacity, changes of which during activity are neglected (see Cole & Curtis 1939). Thus conduction velocity depends upon five quantities of which three, namely, the radius of the fibres, the membrane capacity and the specific internal resistance, have been

measured in some invertebrate nerve and in muscle fibres, while the 'safety factor' is not accurately known, and the active membrane resistance has only been worked out for the squid giant axon. It is clear, however, that according to all these theories the membrane capacity plays an important part in determining conduction speed. In table 6, the values of $C_m^{-1} \times \sqrt{(\text{radius}/R_t)}$ have been listed for squid nerve, crab nerve and frog muscle and compared with the conduction velocities in a large volume of saline. There is a reasonable correlation between the three physical factors and the conduction velocity, though the matter cannot be pursued until the other factors have been clarified.

TABLE 6. CONDUCTION VELOCITIES *IN SITU* AT 20° C

	radius (μ)	C_m (μF/cm.²)	R_t (Ω cm.)	$\dfrac{\sqrt{\text{radius}}}{C_m \sqrt{R_t}}$ (rel. units)	conduction velocity (m./sec.)	data taken from
squid nerve	250	1·1	30	26	25	Curtis & Cole (1938), Hodgkin (1939), Cole & Hodgkin (1939)
crab nerve	15	1·1	60	4·5	4·5	Hodgkin (1939, 1947), this paper
frog muscle	30	4·8	230	0·75	1·6	this paper

(3) *The membrane constants and the end-plate potential in curarized muscle*

When neuro-muscular transmission is blocked by curarine, a nerve impulse produces a subthreshold depolarization of the muscle, the end-plate potential (e.p.p.), which spreads a few mm. along the fibre surface (Eccles *et al.* 1941). This e.p.p. decays much more slowly than the muscle spike, and two alternative explanations have been put forward for this behaviour. According to Coppée (1943), the e p.p. arises from a prolonged activity at the nerve endings and is, in fact, a negative after-potential of the motor end-plate. According to Eccles *et al.* (1941), however, the period of activity during neuro-muscular transmission is very brief, of the order of 4 msec , and the prolonged decline of the e.p.p. is due to the slow dissipation of charge along and across the resting fibre membrane.

If it is true that the depolarizing action of the transmitter is turned 'on' and 'off' within about 4 msec., then it should be possible to reproduce the e p.p. by applying an equally brief subthreshold current pulse to the muscle. As the most accurate information on the spread of the e.p.p. was obtained on whole muscle, this experiment was made on the M. extensor dig. IV, using the circuit diagram of figure 1 (*b*). Tracings of the extrapolar potential are shown in figure 10; they may be compared with the e.p.p. at a junctional focus of the sartorius (taken from Eccles *et al.* 1941, figure 5). The resemblance of the two types of potential is close and becomes even closer if account is taken of differences in temperature and the fact that the rate of decline of the e.p.p. has a Q_{10} of 1·3 (Eccles *et al.* 1941). The potential changes differ appreciably only in the rate of attenuation over the first mm.: the e.p.p. spreads further, probably because the individual nerve endings are scattered over a narrow zone of muscle (Eccles *et al.* 1941, p. 367). There is, however, little doubt that a very

brief influence, such as the subthreshold current pulse shown in figure 10, leaves behind a potential change which decays slowly and is almost indistinguishable from an e.p.p.

In his study of isolated junctions of the M. adductor longus, Kuffler (1942) recorded e.p.p.'s which are quicker than those obtained in whole muscle. This difference may be attributed to two causes: (1) in whole muscle the e.p.p. is recorded
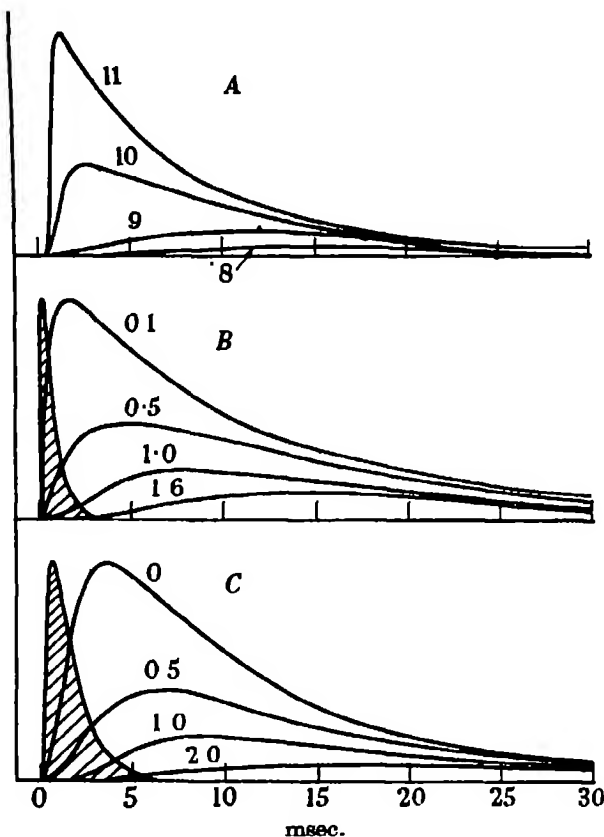
FIGURE 10. End-plate potential of curarized frog muscle (A) and its reconstruction by a brief current pulse (B and C). A, from Eccles *et al.* (1941, figure 5), showing e.p.p. in frog's sartorius at 27·5° C. Distances from pelvic end shown in mm. B and C, M. extensor dig. IV. Extrapolar potentials produced by a brief current pulse which is shown by the shaded area. Distances between leads E_2 and E_5 in mm. B, 22·5° C., $\lambda = 1\cdot04$ mm., $\tau_m = 17$ msec. C, a longer current pulse. 18 5° C., $\lambda = 1\cdot2$ mm., $\tau_m = 25$ msec. The curve at 'zero distance' has been corrected for a small current escape (cf. figure 8).

at a small distance from the majority of the junctions, and (ii) the isolated fibre had presumably a fairly low membrane resistance. If the mean value of τ_m ($= 9$ msec.) obtained on isolated fibres is valid for Kuffler's preparations, we can pursue the analysis of the e.p.p. a step further. In figure 11 the e.p.p. recorded from the junction of a curarized muscle fibre (in paraffin oil) has been reproduced (Kuffler (1942), figure 8). By a simple numerical analysis suggested to me by Professor A. V. Hill and using Hodgkin & Rushton's (1946) equation 4·1 we can now determine the

current pulse which would produce an electrotonic potential, at the electrode, identical with the e.p.p. The result of this analysis (figure 11 A) is a brief pulse which rises and falls within 4 msec. This agrees very well with Kuffler's (1942) conclusion, viz. that the active phase of the transmitter cycle is completed within 4 msec. A similar, but more approximate, analysis has been made for the e.p.p. in a curarized M. extensor dig. IV (figure 11 B). In this case, the 'transmitter pulse' derived in figure 11 A has been adopted and the e.p.p. reconstructed by reversing the above analysis.
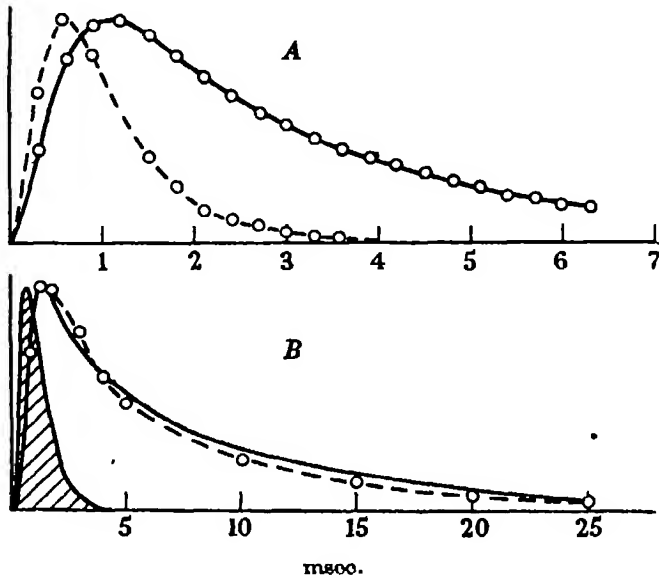
FIGURE 11. A, full line: from Kuffler (1942, figure 8), showing e.p.p. at an isolated junction of M. adductor longus. Broken line analyzed subthreshold current which is equivalent in its action to the neuro-muscular transmitter. Analysis based on $x = 0$, $\tau_m = 9$ msec. Circles show results of the numerical analysis. B, full line e p.p. in a curarized M. extensor dig IV; $\lambda = 0.86$ mm., $\tau_m = 13$ msec. Shaded area: equivalent current pulse, as in A. Circles and broken line: electrotonic potential produced by this pulse at $x = 0.1\lambda$.

Thus, the results of the cable analysis offer strong supporting evidence for the thesis that the active phase of myoneural transmission is brief, and that the e.p.p. is prolonged merely because the muscle membrane can hold electric charge for many milliseconds. There are, however, limitations to the accuracy of the present analysis, for, the depolarization by an electric current does not completely imitate that by a chemical transmitter (see Katz 1942), nor can we reproduce the exact situation of the synapse by an externally applied electrode.

The suggestion of a brief action and prolonged passive decay applies to curarine-treated muscle, but not necessarily to other cases, e g. not to eserinized muscle in which the active period is known to be lengthened (Eccles et al. 1942). Coppée's (1943) evidence was derived largely from experiments with various other drugs, under whose influence the active phase of the transmitter cycle may well have been prolonged.

DISCUSSION

The experiments described in this paper confirm that the electrical properties and reactions of the muscle membrane are very similar, apart from quantitative differences, to those of a non-medullated nerve axon. In view of the different structure and function of the two types of cells, the analogous behaviour of their surface membranes has frequently been questioned. For example, Dubuisson (1942) in a recent review has discussed the possibility that the distribution of ions in muscle fibres is essentially different from that in nerve, and that the bulk of the muscle potassium might be enclosed in the anisotropic disks of the fibrils rather than being dissolved in the cell water. Although such arguments are speculative, one must seriously consider whether the muscle fibre may not be subdivided into smaller 'cable elements', and whether potential changes at the boundaries between myofibrils and sarcoplasm might not conceivably be responsible for the electrotonic potential of the muscle fibre.

It is worth while, therefore, to summarize the evidence which shows that the surface membrane of the muscle fibre is the most probable seat of the observed potential changes.

The structures at which the electrotonic potential (or the e p p) and the action potential of muscle arise, appear to be identical, for not only does the spike develop from the catelectrotonic potential, but the latter is temporarily abolished and gradually rebuilt after the spike has passed (Kuffler 1942; see also § A above) The two voltages do not sum, as they would if they arose at two separate types of membrane in series with one another.

Hence, the argument concerning the site of the membrane applies to the spike as well as to the electrotonic potential. The fact that the spike is an all-or-none reaction which involves the muscle fibre as one unit makes it difficult to believe that the action potential can be built up by numerous smaller units inside the cell. More direct evidence has been obtained recently by Graham & Gerard (1946) who recorded large resting and action potentials when a microelectrode was inserted into a muscle fibre. With this technique the potential across the surface membrane of the fibre must have been recorded, for it is inconceivable that a recording electrode could have penetrated a myofibril without destroying it.

The ratio r_i/r_o in the present experiments is relevant to this problem. In clean muscle bundles r_i/r_o was as low as 0·25, with a mean of 0·47. Assuming that the electrotonic potential arose at the *surface* membrane, r_o and r_i are the resistances, respectively, of the interstitial fluid and the fibre contents. But on the hypothesis that the electrotonic potential arose at the interfaces of myofibrils and sarcoplasm, r_i would be the resistance of the myofibrils and r_o the parallel resistance of sarcoplasm and surrounding Ringer space. According to Dubuisson (1942, p 451) the volume proportions of Ringer space, sarcoplasm, and myofibrils are 14·5 28·5 · 57. If we use these data, the specific resistance of Ringer solution (83 Ω cm. at 22° C) the average resistivity of muscle (157 Ω cm.) and the mean value of r_i/r_o (0·47), we can test the validity of both hypotheses. On the first assumption it can be shown that the specific resistance of the outside fluid would be 71 Ω cm., which is not far from the

correct value of $83\,\Omega$ cm. On the second hypothesis, the external space includes the sarcoplasm, and we can calculate what the specific resistance of 'myofibrils' and 'sarcoplasm' should be to satisfy this hypothesis. The results are 132 and $983\,\Omega$ om. for fibrils and sarcoplasm respectively. That is to say, the fibrils would have to be better conductors than the cell sap if we are to record appreciable potential changes from them. According to Schmitt, Bear, Hall & Jakus (1947) the myofibrils are made up of numerous protein filaments which presumably contribute little to the conduction of current. It is, therefore, more probable that these structures, far from being 'core-conductors', are effectively short-circuited by the potassium-rich fibre water (Boyle & Conway 1941), and that their only contribution to the electrical measurements is that of an insulator which takes up a fraction of the cell volume and thereby increases the value of R_i.

One may reasonably conclude that R_m and C_m represent properties of the surface membrane of the muscle fibres. In table 7 the known membrane constants of several excitable tissues have been listed, they include frog's muscle as well as three types of non-medullated nerve. When surveying the values of R_m one gains the impression that there is a relation between fibre size and membrane conductivity, as pointed out by Hodgkin & Rushton (1946). In comparing frog tissue with that of marine invertebrates, account must be taken of the different salinity of external and internal medium. The conductance of a sq.cm. of membrane is roughly the same in a muscle fibre as in an equally large crustacean nerve fibre, in spite of the fact that the salt solutions which surround the muscle membrane have a 4 times lower conductivity. This would seem to suggest that the surface membrane of muscle is a less effective ion barrier than that of a nerve axon of the same diameter. The membrane capacity, however, is much larger in muscle than in the three non-medullated nerves. It is possible that both differences arise from a single fundamental property: for example, if the muscle membrane were thinner, or more thoroughly impregnated with ions, than the nerve membranes, the differences of R_m and C_m would be explained.

TABLE 7. ELECTRICAL CONSTANTS OF NERVE AND
MUSCLE FIBRES (TEMP. APPROX. 20° C)

fibre type	diameter (μ)	λ (mm.)	τ_m (msec.)	R_m (Ω cm.^{2})	C_m (μF/cm^2)	R_i (Ω cm.)	R_e (Ω cm.)	data from
squid nerve	500	2·5	0·7	700	1	30	22	Curtis & Cole (1938); Cole & Hodgkin (1939)
lobster nerve	75	1·5	2	2000	1	60	22	Hodgkin & Rushton (1946)
crab nerve	30	2	5	5000	1	60	22	Hodgkin (1947), and this paper
frog muscle (bundles of add. magnus)	75	0·7	9	1500	6	200	87	this paper
frog muscle (extensor dig. IV)	45	1·1	18	4000	4·5	250	87	this paper

The conductance R_m^{-1} has been regarded as an index of the total ion density and permeability of the membrane (Cole 1940). This is a reasonable concept, for it seems to imply no other assumption than that the resistance to the transport of ions across the membrane should be the same whether the moving force arises from a concentration gradient or from an electric potential difference. The analogy would fail, however, unless the value of R_m can be measured under conditions in which the membrane behaves truly as a passive conductor. To approach this condition, 'inward' currents were used and the analysis was restricted to the anodic region in which an approximately linear relation between current and voltage was found. Nevertheless, such linearity is not final proof of the 'passive' behaviour of the fibre membrane. It is conceivable—and a model has recently been described which illustrates this point (Katz 1947 a)—that any current, anodic or cathodic, alters the ion permeability of the membrane and leads to a regenerative potential change across it. Thus, until there are direct means, for example, by the use of tracer ions, of testing the relation between electric conductance and ion permeability of the membrane, the theoretical significance of the value of R_m will remain uncertain.

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